Volume 95 NOVEMBER–DECEMBER 1954 Number 3

# The Journal of Infectious Diseases

*Founded 1904 by the John Rockefeller McCormick Memorial Institute for Infectious Diseases*
*under the Editorship of LUDVIG HEKTOEN and EDWIN O. JORDAN*

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PUBLISHED FOR THE JOHN ROCKEFELLER McCORMICK MEMORIAL FUND
OF THE UNIVERSITY OF CHICAGO

THE UNIVERSITY OF CHICAGO PRESS
CHICAGO · ILLINOIS · U.S.A.

# The Journal of Infectious Diseases

*The Journal of Infectious Diseases* is devoted to the publication of original investigations dealing with the general phenomena, causation and prevention of infectious diseases. It is the aim of the journal to occupy a special field and to include only such contributions as bear with reasonable directness on the topics indicated in the title. The biology and chemistry of the various pathogenic organisms, the physiology and anatomy of the morbid processes that they initiate, and the hygienic and sanitary problems to which they give rise are considered to be especially within the scope of the undertaking.

This journal was established in connection with the John Rockefeller McCormick Memorial Institute for Infectious Diseases, by the munificence of the late Mrs. Edith Rockefeller McCormick and the late Mr. Harold F. McCormick. Since 1941 it has been published in connection with the John Rockefeller McCormick Memorial Fund of the University of Chicago.

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The Journal of Infectious Diseases is published bi-monthly, at The University of Chicago Press, 5750 Ellis Avenue, Chicago, Illinois. The subscription price is $7.00 per year; $3.50 a volume; the price of single copies of current volume is $2.00. ¶ Postage is prepaid by the publishers on all orders from the United States, Mexico, Cuba, Puerto Rico, Panama Canal Zone, Republic of Panama, Dominican Republic, Canary Islands, El Salvador, Argentina, Bolivia, Brazil, Colombia, Chile, Costa Rica, Ecuador, Guatemala, Honduras, Nicaragua, Peru, Haiti, Uruguay, Paraguay, Hawaiian Islands, Balearic Islands, Spain and Venezuela. ¶ Postage is charged extra as follows: for Canada and Newfoundland, 40 cents on annual subscriptions (total $7.40); on single copies 10 cents (total $2.10); for all other countries in the Postal Union, 75 cents on annual subscriptions (total $7.75); on single copies 15 cents (total $2.15). ¶ Patrons are requested to make all remittances payable to the University Press in postal or express money orders or bank drafts.

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Business correspondence should be addressed to The University of Chicago Press, 5750 Ellis Avenue, Chicago 37, Illinois.

Communications for the editors and manuscripts should be addressed to Mrs. Dorothy W. Green, Assistant to the Editor, The Journal of Infectious Diseases, 5724 Ellis Avenue, Chicago 37, Illinois.

Entered as second-class matter August 1, 1911, at the post office at Chicago, Illinois, under the Act of March 3, 1879.

Acceptance for mailing at special rate of postage provided for in United States Postal Act of October 3, 1917, Section 1103, amended February 28, 1925, authorized July 5, 1918.

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[PRINTED IN U.S.A.]

J. INFECT. DIS. VOL. 95, NO. 3

# THE PATHOGENICITY OF LEPTOSPIRAS TO FIELD RODENTS IN ISRAEL (A NEW TEST ANIMAL FOR USE IN LEPTOSPIRA RESEARCH)

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Occasional cases of leptospirosis in Israel, in man and cattle, have been observed between 1945 and 1949 and were caused by the serotype grippotyphosa. They occurred sporadically and nearly exclusively in persons whose profession brought them in close contact with sick cattle or cattle carcasses, viz. veterinarians, butchers and cattle owners.

Since 1949 the epidemiological picture has changed. From then on almost every patient had some connection with field work, but no direct contact with cattle.[1,2] The latter situation is comparable with that in several European countries (sugar cane and rice field workers disease, harvest disease, Erbsenpflückerkrankheit).

During winter and spring of 1949 and 1950 an unusually large number of rodents ravaged the fields of Israel. At the same time leptospirosis occurred in man and cattle with extraordinary frequency. The simultaneous nature of these occurrences made it likely that some epidemiologic relationship existed between them.

In those areas where the disease prevailed, Olejnik and Shnyerson[3] as well as the author,[1] found high agglutinin titers in the blood of the local voles, *Microtus guentheri* (Danford and Alston, 1880), against the leptospira type which was isolated from human and bovine patients (*Leptospira grippotyphosa*). Such antibodies, however, were not encountered in voles, caught in places where leptospirosis did not prevail. In other wild rodents (rats, mice, gerbils) leptospira agglutinins were not found.

The present paper deals with an investigation on the susceptibility of field rodents in Israel (excluding the house mouse, *Mus musculus*) to various leptospira serotypes and their probable role in the spread of infection.

## MATERIALS AND METHODS

The materials used for the infection consisted of: (a) pure cultures of several leptospira serotypes and (b) fresh blood, urine and suspensions of organ tissues from spontaneously infected men or animals.

Most of the cultures had been incubated at 30 C during 5 to 7 days prior to use. Slowly growing strains were allowed to remain in the incubator for a longer time, viz., up to 12 days.

No special effort was made to overcome the technical difficulties of counting the number of leptospiras in the inoculum. In most of the cases the cultures used were of a density of 10 to 100 leptospiras in every darkfield picture (dry system

Received for publication November 23, 1953.

The author is greatly indebted to Dr. T. Nobel, pathologist of the Veterinary Institute for the histologic examination of a great number of tissue sections; to Mr. J. Naftali of the Plant Protection Service, Ministry of Agriculture, Tel-Aviv, for the supplying of rodents from the field and Microtus and Meriones of his breeding stock; and to Br. B. Babudieri, Rome, Dr. C. Borg Petersen, Copenhagen, Dr. J. C. Broom, London, Dr. E. Olejnik, Tel-Aviv, Dr. E. Wiesmann, St. Gallen, and Dr. J. W. Wolff, Amsterdam, all of whom kindly put at my disposal leptospira strains of their laboratory collections.

1. van der Hoeden, J. 1951, Bull. Off. Int. Epiz. **36**: 400–409.
2. van der Hoeden, J., Halevi, H. and Dafni, I. 1953, J. Comp. Path. & Therap. **63**: 93–100.
3. Olejnik, E. and Shnyerson, S. 1950, Nature, **155**: 526.

magnification 170×). The comparison of any one reaction in different rodents is valid, however, in that all animals in a single experiment received simultaneously the same inoculum of a given material. The dose of the injection was more or less dependent on the number of organisms per cc, varying between 0.5 and 1 cc in the larger rodents, and 0.3 to 0.5 cc in the mice.

Particulars concerning the origin and age of the leptospira strains used for the experiment are summarized in table 1.

The animals were mostly injected subcutaneously or intraperitoneally, and occasionally along the oral route or in the conjunctival sac. Animals in their early stage of maturity were preferred. In order to exclude the possibility of spontaneous infections in the test animals, samples of blood were taken at random from several of these animals prior to inoculation. In no instance were specific agglutinins or lysins against the leptospira serotypes in question found.

After the test animals died or had been killed, paraffin sections of the kidneys were histologically examined by Dr. T. Nobel (haematoxylin-eosin staining) and leptospiras were traced with the Levaditi's silver impregnation technique.

Cultures were ordinarily made from the kidneys and liver, and occasionally also from the heart blood. Pieces of the organs, measuring a few mm, were aseptically removed with scissors, and by means of a platinum spatula macerated against the wall of a culture tube containing 5 cc of the slightly modified Chang's liquid culture medium. After washing the material into the medium, the tubes were kept in the incubator at 30 C during 3 weeks, meanwhile being repeatedly examined for growth. When, after several transplants, sufficient density of the culture was obtained, their serotype was checked against type specific rabbit antiserum.

In addition to mice, rats and voles, the most common field rodent in Israel is a gerbil species, *Meriones shawi* (Duvernoy, 1842). In a relatively small area of the southern part of the country, between Tel-Aviv and Gaza, another gerbil, *Meriones crassus sacramenti* (Thomas, 1922), is found. The closely related, somewhat smaller *Mer. cr. crassus* (Sündevall, 1842) is a quite common desert species. *Gerbillus pyramidum* (Geof-

TABLE 1.—*Cultures used in the experiments.*

| *Leptospira* species | Strain | Isolated from | | Reference |
|---|---|---|---|---|
| | | Host | Country | |
| *grippotyphosa* | Meissar | Goat | Israel | van der Hoeden, 1951[a] |
| | Shoham | Man | Israel | van der Hoeden, 1952 |
| | Geffen | Man | Israel | Olejnik, 1950[b] |
| | Niederhelfenswil | Man | Switzerland | Wiesmann, 1951 |
| | Moscou V | Man | U.S.S.R. | Kritschewski, 1928[c] |
| *icterohaemorrhagiae* | Betjes | Man | Holland | Wolff, 1952 |
| | Bailey | Man* | England | Broom, 1952 |
| | Field | Pig* | England | Field and Sellers, 1950[d] |
| | Wijnberg | Man | Holland | Schüffner, 1926[e] |
| *canicola* | Rishpon | Dog | Israel | van der Hoeden, 1952 |
| | Sarafand | Dog | Israel | van der Hoeden, 1953 |
| | Kfar Ono | Dog | Israel | van der Hoeden, 1953 |
| | Aldgate | Dog† | England | Broom, 1948 |
| | Utrecht Hond IV | Dog | Holland | Schüffner, 1931[f] |
| *ballum* | S102 | White mouse* | Holland | Schüffner, 1941[g] |
| | Ma 31 | White mouse | Denmark | Borg Petersen, 1952 |
| | Wolf | Man‡ | Israel | Olejnik, 1950[b] |
| | Microtus | Vole‡ | Israel | Olejnik, 1950[b] |
| *sejroe* | M809 | Man‖ | Denmark | Borg Petersen, 1952 |
| | Topo I | *Apodemus* | Italy | Babudieri, 1952 |
| *pomona* | Austoni | Man | Italy | Babudieri, 1952 |

* Passed through guinea pig.
† Passed through hamster.
‡ Passed through white mouse, or originating from white mouse.[e]
‖ Passed through white mouse and guinea pig.

[a] van der Hoeden, J. 1953, J. Comp. Path. & Therap. **63**: 101.
[b] Olejnik, E. and Shnyerson, S. 1950, Nature, **166**: 526; Olejnik, E. and Shnyerson, S. 1952, Tr. Roy. Soc. Trop. Med. & Hyg. **46**: 165; van der Hoeden, J. 1953, Tr. Roy. Soc. Trop. Med. & Hyg. **47**: 364.
[c] Tarassof, S. and Epstein, H. 1928, Bull. Inst. Pasteur, **27**: 928; Tarassof, S. 1931, Ann. Inst. Pasteur, **46**: 222; Walch-Sorgdrager, B. 1939, Bull. Health Organ. L.O.N. **8**: 143.
[d] Field, H. S. and Sellers, K. C. 1951, Vet. Rec. **63**: 87.
[e] Borg Petersen, C. 1938, Acta Conv. tert. trop. Morbis, **1**: 396; Gispen, R. and Schüffner, W. 1939, Zentralbl. f. Bakt. **144**: 427.
[f] Klarenbeek, A. and Schüffner, W. 1933, Nederl. Tydjschr. v. Geneesk. **77**: 4271; Schüffner, W. 1934, Tr. Roy. Soc. Trop. Med. & Hyg. **28**: 7.
[g] Borg Petersen, C. 1944, Acta path. et microbiol. Scandinav. **21**: 594; Wolff, J. W., Bohlander, H. and Ruys, A. C. 1949, Antoni v. Leeuwenhoek, **15**: 1.

froy, 1825) also lives in Israel's southern desert, the Negev, and in the extensive areas of sand dunes along the Mediterranean shore.

The test animals employed in our experiments were: *M. guentheri*, *Mer. shawi*, *Mer. cr. sacramenti*, *Gerbillus pyramidium*, the Syrian golden hamster (*Cricetus auratus*), the guinea pig, and albino mice.

## EXPERIMENTAL INFECTIONS

In table 2 are summarized the reactions which were shown by the rodents mentioned above after injection with *Lept. grippotyphosa*, *Leptospira icterohaemorrhagiae*, *Leptospira canicola*, *Leptospira sejroe*, *Leptospira ballum*, and *Leptospira pomona*, respectively.

Several strains differed considerably in the time which elapsed since their original isolation from the host animal and their employment in this research.

The influence of prolonged cultivation in artificial medium on the virulence of certain strains has been investigated by repeated testing of the transplants.

In a few instances a comparison could be made of the results of infection following the use of original animal material (blood, urine, tissues) containing leptospiras of unimpaired virulence with those of cultures obtained from the same materials.

## SUMMARY AND CONCLUSIONS

1. All species of rodents mentioned in the above investigations proved to be susceptible to infection by various serotypes of leptospiras. Differences in susceptibility were obvious from the morbid symptoms, the mortality, the appearance of jaundice and interstitial nephritis, and the presence of leptospiras in blood and organs. All animals showing icterus died.

2. As a rule in *M. guentheri*, infection did not cause perceptible clinical reactions. Only rarely did illness and death occur (with *Lept. icterohaemorrhagiae* and *Lept. pomona*). In no case was jaundice noticed. Occasionally interstitial nephritis was found upon histological examination, and leptospiras were regularly present in the kidneys. Here they remained for at least as long as 4 months after the animal was infected. It may be concluded that certain serotypes of leptospiras are able to induce a healthy carrier state in *M. guentheri* and that the organisms are shed in its urine.

3. Various leptospira serotypes exert a more pathogenic effect on *Mer. shawi*, *Mer. cr. sacramenti* and *Gerb. pyramidum* than on *M. guentheri*. Almost every animal of those three species of gerbils used in the experiment died within the week following infection either with recently isolated cultures or with fresh material from infected animals. The majority of infected gerbils showed icterus, especially in rapidly developing cases. Like in the vole, jaundice was never noticed in guinea pigs and mice, and even in golden hamsters icterus occurred less constant than in the gerbils.

4. Dropping a culture of *Lept. icterohaemorrhagiae* into the eyes and mouth of *Gerb. pyramidum* and infecting *Mer. cr. sacramenti* along the same routes with *Lept. canicola* proved to be very effective. It must be taken into account that in such cases the number of leptospira organisms entering into the tissues is much smaller than by direct subcutaneous or intraperitoneal injection.

5. Subculturing of leptospiras usually caused rapid reduction and finally even complete loss of virulence. Some strains remained virulent much longer for several species of rodents than other strains of the same serotype. The pathogenicity of very old leptospira strains to the gerbil species was similar to that of freshly isolated strains to *M. guentheri*. When comparing the reactions following injection with fresh animal material with those after injection with culture-suspensions, one should bear in mind that in the former case the number of or-

TABLE 2.—*Experimental infection of different rodents by various leptospira serotypes.*

| Test animal | Material injected | Strain | Age of strain since isolation | Route of infection | Number of days after infection: Died | Killed | Culture from: Blood | Liver | Kidney | Leptospiras in sections of: Liver | Kidney | Icterus | Interstitial nephritis |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | *Lept. grippotyphosa* | | | | | | | | |
| *M. guentheri* | Culture | Meissar | 40 days | s.c. | | 8 | − | − | + | − | − | − | + |
| | Culture | Meissar | 40 days | s.c. | | 18 | − | − | + | + | + | − | + |
| | Culture | Meissar | 40 days | s.c. | | 46 | | − | + | (+) | (+) | − | − |
| | Culture | Meissar | 40 days | s.c. | | 63 | | − | + | − | (+) | − | |
| | Culture | Shoham | 5 days | i.p. | | 115 | | | − | | + | − | |
| *Mer. shawi* | Culture | Meissar | 40–50 days | s.c. (5) | 4–5 | | + | + | + | + | + | + | + |
| | Culture | Meissar | 198 days | s.c. | | 32 | | + | + | − | + | − | + |
| | Culture | Meissar | 305 days | s.c. | 5 | | | | | − | + | + | − |
| *Mer. cr. sacramenti* | Culture | Meissar | 54 days | s.c. (2) | 4–5 | | + | + | + | + | + | + | + |
| | Culture | Meissar | 332 days | s.c. | 11 | | | | + | + | + | − | − |
| | Culture | Shoham | 5 days | i.p. | 4 | | | + | − | + | + | + | |
| | Culture | Niederh-elfenswil | ± 1 year | i.p. | 9 | | | | | + | + | ± | + |
| | Culture | Geffen | 2½ years | s.c. | 4 | | + | + | + | + | + | + | − |
| | Culture | Moscou V | 22 years | i.p. | | 160 | | − | − | − | + | − | − |
| | Human urine | Shoham | | i.p. | 21 | | − | | + | + | + | + | − |
| | Mouse urine | Geffen | | i.p. | | 108 | | − | + | − | + | − | + |
| *Gerb. pyramidium* | Culture | Meissar | 39 days | s.c. (4) | 6–7 | | | + | + | + | + | + | + |
| | Culture | Meissar | 54 days | s.c. | 5 | | | | | + | + | + | + |
| | Culture | Meissar | 170 days | s.c. | 7 | | | | | − | − | ± | + |
| | Culture | Meissar | 54 days | mouth | 10 | | + | + | + | − | + | ± | + |
| | Culture | Meissar | 54 days | eye | 11 | | | + | − | | | ± | |
| Syrian golden hamster | Culture | Meissar | 40 days | s.c. | | 8 | + | + | + | − | − | − | + |
| | Culture | Meissar | 40 days | s.c. | | 19 | − | + | + | − | + | − | + |
| | Culture | Meissar | 40 days | s.c. | | 46 | | − | − | | | − | |
| | Culture | Meissar | 50 days | i.p. | | 7 | | | + | + | + | − | |
| | Culture | Meissar | 50 days | i.p. | 10 | | | | | + | + | − | + |
| | Culture | Shoham | 5 days | i.p. | 6 | | | | | + | + | + | + |
| | Goat blood | Meissar | | i.p. | 34 | | | + | + | + | + | − | + |
| | Goat milk | Meissar | | i.p. | 11 | | | | | | + | − | |
| | Goat urine | Meissar | | i.p. | 17 | | | | | + | + | ± | + |
| | Goat liver and kidney | Meissar | | i.p. | 11 | | | | | | + | | |
| | Test calf urine* | Meissar | | s.c. | 7 | | | − | − | + | + | + | + |
| | Test calf kidney* | Meissar | | s.c. | 8 | | | + | + | | | + | |
| | Test calf liver* | Meissar | | s.c. | 15 | | | | − | − | + | + | |
| | Human blood | Shoham | | i.p. | 31 | | | + | − | | | + | |

( ) = number of animals tested.
± = subicteric
(+) = suspicious granules, but no distinct leptospiral structures.
− = negative result
+ = positive result.
Blank space = not examined (in most cases due to putrefaction).
s.c. = subcutaneus
i.p. = intraperitoneal.
* Test calf slaughtered 24 days after subcutaneous infection of *Lept. grippotyphosa*, strain Meissar.

TABLE 2—*Continued*

| Test animal | Material injected | Strain | Age of strain since isolation | Route of infection | Number of days after injection: Died | Killed | Culture from: Blood | Liver | Kidney | Leptospiras in sections of: Liver | Kidney | Icterus | Interstitial nephritis |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Guinea pig | Culture | Meissar | 50 days | i.p. | | 8 | | | + | + | + | − | + |
| | Culture | Meissar | 50 days | i.p. | | 15 | | | + | − | + | − | − |
| | Culture | Meissar | 50 days | i.p. | | 21 | | | + | − | − | − | − |
| | Culture | Meissar | 50 days | i.p. | | 28 | | | − | − | − | − | − |
| | Goat blood | Meissar | | i.p. | | 34 | | | | | + | − | |
| | Goat milk | Meissar | | i.p. | | 34 | | | + | + | + | − | + |
| | Goat urine | Meissar | | i.p. | | 34 | | | | + | + | − | + |
| | Goat liver and kidney | Meissar | | i.p. | 11 | | | | | − | + | − | + |
| Albion mouse | Culture | Meissar | 13 days | i.p. | | 190 | | | + | − | + | − | + |
| | Culture | Meissar | 27 days | i.p. | | 21 | | − | + | − | − | − | + |
| | Culture | Meissar | 50 days | i.p. (5) | | 7–28 | | | + | + | + | − | + |
| | Culture | Meissar | 185 days | i.p. | | 21 | | − | − | − | − | − | + |
| | Culture | Geffen | 2½ years | i.p. | | 21 | | − | + | − | + | − | + |
| | Goat blood | Geffen | | i.p. | 28 | | | | | + | + | − | + |
| | Goat milk | Geffen | | i.p. | 33 | | | + | + | + | + | − | + |
| | Goat urine | Geffen | | i.p. | 34 | | | | | + | + | − | + |
| | Goat liver and kidney | Meissar | | i.p. | 33 | | | + | + | + | + | − | + |
| | | | | | *Lept. icterohaemorrhagiae* | | | | | | | | |
| *M. guentheri* | Culture | Betjes | ±6 weeks | i.p. | 5 | | − | + | + | + | + | ± | |
| | Culture | Bailey | ±4 weeks | i.p. | | 120 | | | − | + | + | − | |
| *Mer. shawi* | Culture | Betjes | ±6 weeks | i.p. | 3 | | + | + | + | + | + | + | |
| | Culture | Bailey | ±4 weeks | i.p. | 3½ | | | | | | | ± | |
| | Culture | Field | ±2 years | i.p. | 4 | | | | | + | + | + | − |
| *Mer. cr. sacramenti* | Culture | Betjes | ±6 weeks | i.p. | 3½ | | | | | − | + | + | + |
| | Culture | Bailey | ±4 weeks | i.p. | 4 | | + | + | + | + | + | ± | − |
| | Culture | Field | ±2 years | i.p. | 3½ | | | | | + | + | + | + |
| | Culture | Wijnberg | 26 years | i.p. | | 160 | | | | − | − | − | − |
| Syrian golden hamster | Culture | Betjes | ±6 weeks | i.p. | 5 | | | | | + | + | + | + |
| | Culture | Bailey | ±4 weeks | i.p. | 7 | | − | + | | + | + | + | − |
| | Culture | Field | ±2 years | i.p. | 8 | | | − | + | + | + | ± | + |
| | | | | | *Lept. canicola* | | | | | | | | |
| *M. guentheri* | Culture | Sarafand | 90 days | i.p. | | 35 | | − | − | − | + | − | + |
| | Culture | Aldgate | 4 years | i.p. | | 120 | | | − | − | + | − | − |
| | Dog urine | Rishpon | | i.p. | | 110 | | − | − | + | + | − | − |

TABLE 2—*Continued*

| Test animal | Material injected | Strain | Age of strain since isolation | Route of infection | Number of days after infection | | Culture from | | | Leptospiras in sections of | | Icterus | Interstitial nephritis |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Died | Killed | Blood | Liver | Kidney | Liver | Kidney | | |
| | Culture | Sarafand | 90 days | s.c. | 11 | | | | | − | + | | + |
| *Mer. shawi* | Culture | Sarafand | 90 days | eye | | 38 | | − | − | − | − | − | − |
| | Culture | Sarafand | 90 days | mouth | | 38 | | − | − | − | − | − | − |
| | Culture | Aldgate | 4 years | i.p. | 6 | | | | + | | | ± | |
| | Culture | Kfar Ono | 20 days | i.p. | 6 | | + | + | + | + | + | + | + |
| | Culture | Sarafand | 12 days | i.p. | 5 | | | | + | | | ± | |
| | Culture | Sarafand | 62 days | s.c. | | 23 | − | − | − | + | + | − | + |
| | Culture | Sarafand | 62 days | eye | 8 | | − | + | + | + | + | − | + |
| *Mer. cr. sacramenti* | Culture | Sarafand | 62 days | mouth | 7 | | | | | + | + | − | + |
| | Culture | Aldgate | 4 years | s.c. | 5½ | | | | + | + | + | + | + |
| | Culture | Utrecht HIV | 20 years | s.c. | | 160 | | | | − | + | − | − |
| | Dog urine | Rishpon | | i.p. | 9 | | + | | | + | + | − | + |
| | Dog urine | Mikveh | | i.p. | 7 | | + | + | − | | | ± | |
| Syrian golden hamster | Dog urine | Rishpon | | i.p. | 12 | | | − | + | + | + | − | + |
| | | | | | *Lept. sejroe* | | | | | | | | |
| *M. guentheri* | Culture | M809 | 5 months | i.p. | | 120 | | − | + | + | + | − | + |
| | Culture | Topo I | Several months | i.p. | | 120 | | | − | − | + | − | |
| *Mer. shawi* | Culture | M809 | 5 months | i.p. | 4 | | | + | + | + | + | + | + |
| | Culture | Topo I | Several months | i.p. | 6 | | − | − | − | (+) | + | − | + |
| *Mer. cr. sacramenti* | Culture | M. 809 | 5 months | i.p. | 4 | | | + | + | + | + | + | − |
| | Culture | Topo I | Several months | i.p. | 8 | | | | | (+) | − | ± | − |
| Syrian golden hamster | Culture | M. 809 | 5 months | i.p. | 5 | | + | + | + | + | + | + | + |
| | Culture | Topo I | Several months | i.p. | 5 | | + | + | + | + | + | + | + |
| | | | | | *Lept. ballum* | | | | | | | | |
| *M. guentheri* | Culture | Ma 31 | 10 weeks | i.p. | | 120 | | − | − | − | + | − | − |
| *Mer. shawi* | Culture | Ma 31 | 10 weeks | i.p. | 6 | | + | + | + | + | + | − | − |
| | Culture | Ma 31 | 10 weeks | i.p. | 15 | | | | | − | + | ± | + |
| *Mer. cr. sacramenti* | Culture | Wolf | 2 years | i.p. | 7 | | | | | + | + | ± | − |
| | Culture | Microtus | 2 years | i.p. | | 160 | − | − | | − | + | − | − |
| | Culture | S102 | 11 years | i.p. | | 160 | | − | − | − | + | − | − |
| Hamster | Culture | Ma 31 | 10 weeks | i.p. | | 90 | | | | | | − | |
| | Culture | Wolf | 2 years | i.p. | | 21 | | + | + | + | + | − | + |
| Mouse | Culture | Microtus | 2 years | i.p. | | 21 | | − | + | − | + | − | + |
| | Culture | S102 | 11 years | i.p. | | 21 | | − | − | + | + | − | + |
| | | | | | *Lept. pomona* | | | | | | | | |
| *M. guentheri* | Culture | Austoni | Several months | i.p. | 8 | | | − | − | + | + | − | + |
| *Mer. shawi* | Culture | Austoni | Several months | i.p. | 4 | | | | | + | + | ± | − |
| *Mer. cr. sacramenti* | Culture | Austoni | Several months | i.p. | 5½ | | | + | − | + | + | + | − |

ganisms is much smaller than in the latter. It seemed that the leptospiras present in the animal tissues were more virulent than those in the primary culture isolated from these organs.

6. Young Syrian hamsters have been recommended by several authors as suitable test animals for diagnostic and research work with regard to *Lept. icterohaemorrhagiae* and *Lept. canicola*. Our data show that *Lept. grippotyphosa* and *Lept. sejroe* are also pathogenic for this rodent.

7. In view of their great susceptibility to several leptospira serotypes, *Mer. shawi* and *Mer. cr. sacramenti* are excellent test animals in leptospira research. The last mentioned species is the one of special preference. It is docile, can be easily handled, does not bite, is mute, moves rather slowly, does not jump, and its size is equal to that of a large rat (approximate weight 150 to 250 g). Raising under laboratory conditions is very successful.

8. Probably in nature leptospirosis in gerbils is self limiting because these animals succumb soon after infection. In contrast with this, *M. guentheri* survives infection. Infected voles remain active and become chronic leptospira shedders with the urine for protracted periods. In consequence of their lower susceptibility voles therefore play a greater part in the epidemiologic spread of infection than do the gerbils.

9. Several authors are of the opinion that low pathogenicity of leptospires for certain animal species (e.g., *Lept. icterohaemorrhagiae* for rats) is the result of adaptation of the microbe to the host organism. The well adapted organisms multiply in the tissues without causing obvious damage.[5] Our experience does not concur with this view. "Normal hosts" seem to be those animal species which, due to their low natural susceptibility, remain apparently healthy and are excretors of leptospiras.

If asymptomatic infections were due to adaptation to the parasites it can hardly be understood why *M. guentheri*, for example, showed marked resistance to various leptospira serotypes which have not been encountered locally, whereas all of the serotypes were highly virulent for the gerbils of the same area.

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# THE MOUSE ERYTHROCYTE HEMAGGLUTININ OF FELINE PNEUMONITIS VIRUS

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Haig and Hilleman (1950) have demonstrated a mouse erythrocyte hemagglutinin in the allantoic fluid of embryonated eggs infected with the meningopneumonitis strain of ornithosis virus. This report describes the production of a similar hemagglutinin by another member of the psittacosis-lymphogranuloma group, the feline pneumonitis virus of Baker (1942). The hemagglutinin produced by the growth of feline pneumonitis virus in the allantoic cavity of embryonated eggs was completely separated from the infective virus particles and could be concentrated by differential centrifugation or by adsorption on the flocculent precipitate produced by freezing and thawing infected allantoic fluid. Simultaneous titration of hemagglutinin and infectivity at different times during growth of feline pneumonitis virus in the allantoic cavity indicated that production of the hemagglutinin is dependent upon, but not strictly proportional to, the multiplication of the virus.

## MATERIALS AND METHODS

The strain of feline pneumonitis virus used in these experiments has been described by Moulder and Weiss (1951). For the production of the mouse erythrocyte hemagglutinin, infected yolk sac emulsions were diluted in veal infusion broth and inoculated in 0.25 ml amounts into the allantoic cavity of 8-day embryonated eggs. The highest titers of hemagglutinin were obtained when the virus inoculum contained approximately $10^6$ to $10^7$ $LD_{50}$'s. The incubation period was 4 days at 37 C. The eggs were refrigerated overnight at 4 C before harvesting and the collected fluids maintained at 4 C for all subsequent operations.

Hemagglutinin titer was determined by the method of Hilleman (1951). McIlvaine's buffer (Lange, 1939), as used in this procedure, was kept at pH 7.0. The mouse blood was collected in a small flask containing Alsever's solution (Kabat and Mayer, 1948). The cells were washed by centrifugation at 2000 r.p.m. for 10 minutes in 0.9 NaCl. After three washings, a 1% cell suspension was prepared in McIlvaine's buffer diluted 1:4 with 0.9% NaCl. All hemagglutinin titrations were performed at room temperature.

The virus concentration of the infected allantoic fluids was obtained by inoculating 6-day embryonated eggs via the yolk sac with suitable dilutions of the fluids. Virus titer was then determined either as $LD_{50}$'s per ml (Moulder and Weiss, 1951) or by the average day of death of the infected chick embryos.

The range of centrifugal fields required for the separation and concentration of the hemagglutinin were provided by the use of three centrifuges. Low speed work was done with a size 1 International Centrifuge with a horizontal head. Deposition of the virus from allantoic fluid was accomplished with a Servel SS-1 angle centrifuge. Ultracentrifugal fields were provided by a Spinco preparatory ultracentrifuge. All three centrifuges were maintained at a temperature of 0 to 4 C.

## RESULTS

*Properties of the mouse erythrocyte hemagglutinin.*—Inoculation of 8-day embryonated eggs via the allantoic cavity with $10^6$ to $10^7$ $LD_{50}$'s of feline pneumonitis virus produced a mouse erythrocyte hemagglutinin in the allantoic fluid. After an incubation period of 4 days, the individual allantoic fluids showed a range of hemagglutinin titers from 0 to 1:32. Only a small proportion of the fluids in any group of eggs at-

Received for publication December 14, 1953.

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tained a titer of 1:32, and only occasionally a titer of 1:64 was reached. Maximum hemagglutinin activity is maintained for only a short time. Storage for 48 hours at 4 C resulted in a 50% drop in titer.

In its reaction to physical and chemical agents, the hemagglutinin behaved very much like the hemagglutinin of meningopneumonitis virus (table 1). (In these experiments, the relatively high hemagglutinin titer of 1:128 was obtained by a method of concentration to be described below.) In the presence of formalin and phenol the hemagglutinin activity was destroyed completely. Exposure to a temperature of 56 C resulted in an 8-fold drop in titer after 30 minutes. All of the titrations were made in pH 7.0 buffer, since it was found that wide deviations on both sides of the neutrality point resulted in little or no hemagglutination of the mouse red blood cells. These effects are strikingly similar to those described for the meningopneumonitis virus hemagglutinin (Hilleman et al, 1951).

The purpose of the virucidal ultraviolet radiation was to determine whether the presence of the viable virus was a necessary fact or in hemagglutination. Infected allantoic fluid was exposed to ultraviolet light under the conditions employed by Brown (1950) for complete destruction of virus infectivity. The maintenance of the same titer before and after exposure to the ultraviolet would indicate that it is not necessary for hemagglutination. Further evidence for the independence of the hemagglutination reaction on the presence of the virus particle is furnished by the complete separability of the hemagglutinin and the virus.

TABLE 1.—*The effect of physical and chemical agents on the hemagglutinin of feline pneumonitis virus.*

| Treatment | Hemagglutinin titer |
|---|---|
| Control | 1:128 |
| 1% phenol at 25 C for 30 minutes | 0 |
| 1% formalin at 25 C for 30 minutes | 0 |
| Waterbath at 56 C for 30 minutes | 1:16 |
| Ultraviolet radiation* | 1:128 |

* Fluid exposed in a 1 mm layer to a GE Germicidal lamp for 3 minutes at a distance of 6 inches.

*Separation of hemagglutinin from the virus particle.*—Centrifugation of infected allantoic fluid at speeds sufficient to sediment the virus resulted in no loss of hemagglutinating activity in the supernatant. The proportion of the total virus content of the allantoic fluid sedimented by centrifugation was determined by comparing the infectivity of the supernate with that of the starting material. Allantoic fluid with a hemagglutinin titer of 1:32 was selected as the starting substance. Total virus content per ml of fluid was measured by titration in chick embryos. The material was then centrifuged at 5000×g for 1 hour. The supernatant was collected, titrated for hemagglutinating activity and $LD_{50}$ content, and recentrifuged at 10,000×g for 1 hour. Titration of hemagglutinin and infectivity was repeated on this final supernatant. The above procedure was done with two different pools. The results of this experiment (table 2) indicate that the virus and hemagglutinin are completely separable. Removal of 99.9% of the virus had no effect on the hemagglutinin titer which remained unchanged. Titration of the sedimented

TABLE 2.—*The effect of removal of feline pneumonitis virus from infected allantoic fluid on the original hemagglutinin titer.*

| Allantoic fluid | Log $LD_{50}$ per ml | Hemagglutinin titer | Percentage of total virus |
|---|---|---|---|
| Pool 1 | | | |
| Starting material | 7.71 | 1:32 | 100.00 |
| Supernate 1 (5000×g 1 hour) | 4.60 | 1:32 | 0.078 |
| Supernate 2 (10,000×g 1 hour) | 4.09 | 1:32 | 0.024 |
| Pool 2 | | | |
| Starting material | 7.08 | 1:32 | 100.00 |
| Supernate 1 (5000×g 1 hour) | 4.68 | 1:32 | 0.24 |
| Supernate 2 (10,000×g 1 hour) | 3.93 | 1:32 | 0.070 |

TABLE 3.—*Sedimentation of feline pneumonitis virus hemagglutinin from allantoic fluid stored at −70 C and 4 C.*

| Centrifugal treatment | Hemagglutinin per ml of fluid | |
|---|---|---|
| | Stored at −70 C | Stored at 4 C |
| Original allantoic fluid pool, 10 ml total | 1:16 | 1:16 |
| I. Contrifugation 5000 ×g 60 min, 4 C | | |
| Supernate | 0 | 1:16 |
| Sediment | 1:64 | 0 |
| II. Centrifugation 100,000 ×g 75 min, 4 C | | |
| Supernate (upper 9 ml) | 0 | 0 |
| Sediment (bottom 1 ml) | 1:8 | 1:64 |

virus for hemagglutinin activity indicated that the hemagglutinating factor was not sedimented by the centrifugal fields employed.

*Concentration of hemagglutinin.*—Because of the low titers of hemagglutinin in allantoic fluid, means of concentrating the activity were investigated. Increased hemagglutinin titers per unit volume of fluid were obtained by the following two procedures (table 3):

1. Ten ml of infected allantoic fluid, which had been stored at 4 C for 24 hours, were first centrifuged at 5000 ×g for 1 hour. The original hemagglutinin titer (1:16) was retained in the supernatant. The sediment, resuspended in 1 ml buffer pH 6.9, showed no hemagglutinating activity. The supernatant was centrifuged at an average 100,000 ×g for 75 minutes with no discernible pellet formation. The lower 1 ml titered 1:64, representing a 4-fold increase in hemagglutinin concentration per unit volume of allantoic fluid. This amounts to approximately 40% recovery as, theoretically, complete sedimentation of the hemagglutinin would have shown a 10-fold increase per unit volume over the titer of the starting material. However, on the basis of the serial dilutions used in the hemagglutinin titrations, the deviation between experimental and theoretical recovery amounts to only a one tube difference in titer.

2. The second method utilizes a concentration procedure which has been used previously with the hemagglutinin of influenza virus (Hare, McClelland and Morgan, 1942). The hemagglutinin of both viruses adsorbs on the flocculent precipitate which forms in allantoic fluid thawed to 4 C from the frozen state. The precipitate is readily sedimented in low centrifugal fields and carries with it the hemagglutinating activity of the infected fluid. In this experiment, 10 ml of infected allantoic fluid previously stored for 24 hours at −70 C, were thawed and then maintained at 4 C. The resulting precipitate was thoroughly mixed in the allantoic fluid and the suspension titrated. The hemagglutinin titer was 1:16. Centrifugation at 5000 ×g for 1 hour brought down the virus and the precipitate. The sediment was resuspended in 1 ml buffer pH 6.9, and showed hemagglutinin titer of 1:64 which again represented a 40% recovery. The supernatant had no demonstrable activity. Recentrifugation of the supernate at 100,000 ×g concentrated the small amount of unadsorbed hemagglutinin into the bottom ml of fluid which titrated 1:8. The upper 9 ml were negative for hemagglutinin. Continued work with this method of concentration has shown that the flocculent precipitate can be readily sedimented in a horizontal centrifuge at 300 to 500 ×g with almost complete removal of hemagglutinin from the supernate and a resultant increased titer in the sediment. The prime disadvantage to this method of concentration is that the hemagglutinin remains associated with the components of the precipitate and elution does not appear to take place readily in the buffer. With moderate dilution (1:10) the precipitate goes back into solution at room temperature at pH 7.0. For the purpose of extracting a determinable hemagglutinin concen-

tration from allantoic fluids of low titer, the method is simple and rapid.

One of the factors responsible for the high percentage of allantoic fluids showing absence of hemagglutinating activity is concerned with the relative concentration of hemagglutinin in the infected fluid. In a group of 29 eggs, 18, or 62%, were negative for hemagglutinin when the allantoic fluids were titrated. Processing the total volume of each of the negative samples by the adsorption method and resuspending the sedimented flocculation in 1 ml of buffer showed that ten of these fluids were positive for hemagglutinin. The activity was present in the original fluid but in quantities not detectable except by concentration. The production of hemagglutinin by the feline pneumonitis virus, therefore, is not an all or none phenomenon, but is characterized by a quantitative variation in the amounts produced.

*Relation of virus growth to hemagglutinin titer.*—Although the hemagglutinin and the virus particle are completely separable, the production of this factor is dependent upon the growth of the virus in the allantoic cavity. This was demonstrated by determining the rise in hemagglutinin titer and in virus infectivity in allantoic fluid collected from infected eggs over a period of 4 days. A sufficient number of 8-day embryonated eggs was inoculated with feline pneumonitis virus via the allantoic cavity. Allantoic fluid was pooled from 5 eggs on the 2nd, 3rd, 4th and 5th days after inoculation. The $LD_{50}$ titer of each pool was determined by chick embryo titration. To detect low concentrations of hemagglutinin, particularly in the early stages of infection, the total hemagglutinin content in 5 ml of each allantoic pool was concentrated by the adsorption method described above. The data obtained from this experiment are shown in table 4. During the observed period of time, the titer of both virus and hemagglutinin rises. A 100-fold increase in virus titer between the 2nd and 3rd day is accompanied by the production of 0.8 units of hemagglutinin per ml of allantoic fluid. Between the 3rd and 4th day virus titer increases 4-fold and the hemagglutinin titer 8-fold. There is no apparent increase in virus between the 4th and 5th, day, during which time the hemagglutinin titer is again doubled. On the basis of this one experiment it is difficult to evaluate the significance of the dissimilar increment rates exhibited by the virus and hemagglutinin. In general, the production of the hemagglutinin appears to be closely allied to the growth of the virus in the allantoic cavity.

TABLE 4.—*Infectivity titer and hemagglutinin titer of allantoic fluid collected on the indicated days after allantoic cavity inoculation of 8-day embryonated eggs with feline pneumonitis virus.*

| Days after inoculation | Volume of allantoic fluid (5 eggs) | Log $LD_{50}$/ml | Units of hemagglutinin per ml |
|---|---|---|---|
| 2nd | 13.0 | 5.28 | 0.00 |
| 3rd | 15.0 | 7.35 | 0.80 |
| 4th | 16.0 | 7.96 | 6.40 |
| 5th | 26.0 | 7.82 | 12.80 |

A similar situation, regarding the disproportionality of virus and hemagglutinin content in allantoic fluid, arises when the fluids with different hemagglutinin titers are titrated for virus content. A group of eggs were inoculated with a 0.25 ml inoculum of feline pneumonitis virus containing $10^6$ $LD_{50}$'s. After 4 days incubation, the allantoic fluid from each egg was collected and individually titrated for hemagglutinin content. The infected allantoic fluids, with hemagglutinin titers ranging from 0 to 1:32, were diluted by a constant factor of 1000 and inoculated into the yolk sacs of 6-day embryonated eggs. Ten eggs were used for each fluid and

TABLE 5.—*The average days of death of chick embryos inoculated with allantoic fluids ($10^{-3}$ dilution) having different hemagglutinin titers.*

| Number of allantoic fluid titrations | Hemagglutinin titer | Mean average day of death ± standard error of mean |
|---|---|---|
| 13 | 0 | 5.4±0.1 |
| 6 | 1:4 | 5.3±0.1 |
| 9 | 1:8 | 5.4±0.11 |
| 7 | 1:16 | 5.4±0.08 |
| 5 | 1:32 | 5.2±0.14 |

the average day of death determined. As is shown in table 5, the average days of death of the infected embryos are approximately the same, indicating equal virus content in all of the infected fluids regardless of the amount of contained hemagglutinin. Equal virus content might be expected because of the same virus inoculum and time of incubation used in the preparation of the infected allantoic fluid. The variation in hemagglutinin titer cannot be explained on that basis.

## DISCUSSION

The mouse erythrocyte hemagglutinin of feline pneumonitis virus appears to be similar to the meningopneumonitis virus hemagglutinin on the basis of lability, reaction to physical and chemical agents, maximum titers attained, optimum pH of hemagglutination reaction and the complete separability of the activity from the virus particle. In ultracentrifugal fields the feline pneumonitis hemagglutinin could be concentrated in the lower 10% of the virus-free allantoic fluid. A similar sedimentation of meningopneumonitis virus hemagglutinin was not performed by Hilleman et al (1950), but it was shown that some migration of their hemagglutinin occurred in a more moderate (30,000 to 40,000×g) centrifugal field. Preliminary experiments with another member of the psittacosis-lymphogranuloma group of viruses, murine pneumonitis virus, have indicated that this virus also gives rise to a hemagglutinin of mouse erythrocytes. In all probability other viruses in the psittacosis-lymphogranuloma group can produce a similar hemagglutinin, provided the infective agent is inoculated in a massive dose and undergoes sustained growth in the allantoic cavity of the chick embryo.

With respect to solubility and separability from the virus particle, the hemagglutinin of feline pneumonitis resembles the vaccinia and ectromelia virus hemagglutinins (Burnet and Boak, 1946). The hemagglutinin of the pox viruses are also dissociated without loss of infectivity (Smadel, 1952). If the feline pneumonitis virus hemagglutinin represents an integral portion of the elementary body surface, the amount of this substance should vary proportionately with the virus concentration, at least in the freshly collected fluid. The increment rate experiment and the study of virus concentration versus hemagglutinin titer do not give evidence that any such relationship exists. A similar absence of proportionality has been reported between vaccinia virus titer and the amount of hemagglutinin produced (Hirst, 1952). However, there are a number of factors which may account for the lack of correlation between virus concentration and hemagglutinin titer. The first is the comparatively low titer of hemagglutinin obtained in the allantoic fluid. The entire range of observed hemagglutinin titer from 0 to 1:32 could encompass a variation in virus content so small as to be practically indistinguishable by the methods of virus titration employed. If, in addition, the $LD_{50}$ and hemagglutinin titrations each vary by a factor of 2, the probability of obtaining such disproportional results is within the limits of error of the methods used, even though there is actually strict correlation between the two entities. A second factor is the pres-

ence of a hemagglutinin inhibitor in infected allantoic fluid which can be demonstrated to exist in variable amounts in the fluid samples and whose presence accelerates the sedimentation of mouse erythrocytes in suspension. Sufficient experimental work has not been done as yet to evaluate precisely the effect of such an inhibitor on the absolute hemagglutinin content in allantoic fluid. However, its presence could markedly lower the observed hemagglutinin titer from the true value. The third and final point to be considered concerns the relative lability of both virus and hemagglutinin. As with other viruses, such as the influenza virus, the feline pneumonitis virus hemagglutinin activity does not depend upon the viability of the infective agent. In the growth experiment, only the concentration of the living virus was determined. The lack of strict proportionality in the rate of production of virus and hemagglutinin may be the result of an accumulation of hemagglutinin from the nonviable virus particles. There are unknown variables such as the relative rates of both hemagglutinin and virus decay during growth that complicate the problem of deriving the absolute quantities of both substances produced. Because of these various possibilities which could account for the anomalous virus-hemagglutinin relationship found, the feline pneumonitis virus hemagglutinin may still represent an integral, yet dissociable portion of the elementary body.

## SUMMARY

Growth of feline pneumonitis virus in the allantoic cavity of embryonated eggs produces a mouse erythrocyte hemagglutinin similar to the meningopneumonitis virus hemagglutinin described by Haig and Hilleman (1950).

Virtually complete separation of the hemagglutinin from the virus particle was achieved by sedimentation of the infective agent. On the basis of chick embryo titrations, over 99.9% of the virus was removed from the infected allantoic fluid with retention of the original hemagglutinin titer in the supernatant.

The hemagglutinin of feline pneumonitis virus could be concentrated by ultracentrifugation or by an alternative method involving adsorption of the activity on allantoic fluid precipitate.

The rise in titer of both virus and hemagglutinin in the allantoic fluid of infected embryos was studied. Production of hemagglutinin was dependent upon the growth of the virus in the allantoic cavity, though the increment rates were not strictly proportional. Infected allantoic fluids with different hemagglutinin titers were found to have approximately the same virus concentration.

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# HEPATIC FUNCTION AND OTHER PHYSIOLOGIC STUDIES IN MONKEYS WITH EXPERIMENTAL PNEUMONIC PLAGUE

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Specific antibiotic therapy is highly effective in patients with pneumonic plague if given within a few hours of onset of disease; however, when instituted after 20 hours of disease it fails to prevent death, even though bacterial infection is brought under control.[1] This suggests that profound physiologic disturbances may take place as infection proceeds and that these are not promptly alleviated when bacterial multiplication ceases. Experimentally a substance can be extracted from plague bacilli which is lethal to rats, mice and monkeys, causing increased vascular permeability, shock and hepatic necrosis in rats and mice.[2,3] Similar pathologic changes, notably vascular engorgement and parenchymatous liver changes, have been observed at autopsy in pneumonic plague in man.[4] Since clinical observations of pneumonic plague in man and animals have been limited to signs and symptoms, the present studies on monkeys experimentally infected with *Pasteurella pestis* were undertaken as exploratory attempts to learn some of the more obvious physiologic abnormalities that may occur during the course of this infection.

## MATERIALS AND METHODS

Either pneumonic or bubonic plague was induced in *Macaca rhesus* monkeys weighing 2 to 5 kg with the virulent Indian strain 195/P of *Past. pestis*. Pneumonic plague was established by lightly anesthetizing monkeys with nembutal and delivering 100 to 100,000 organisms in 1 ml of diluent, consisting of 10% monkey serum in physiologic saline solution, through a polyethylene tube placed in the trachea (for additional details, see Ehrenkranz and Meyer[5]); bubonic plague was produced by intracutaneous inoculation of 1,800,000,000 *Past. pestis*.

Rectal temperatures of all experimental animals were taken twice daily. Samples of blood were obtained daily for culture when the temperature was 40 C or more. The following cultures were made: 0.1 ml of blood was spread over each of 1 to 3 blood-agar plates for determining the extent of bacteremia, and in addition 0.5 ml was inoculated into beef-heart infusion broth. Liver biopsy for bacterial culture and liver function tests were performed on monkeys with bubonic plague, and in addition to these studies in monkeys with pneumonic plague, total eosinophil counts, hematocrit and blood pressure recordings were made.

Received for publication February 1, 1954.

The technical assistance of Robert Muir is gratefully acknowledged.

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TABLE 1.—*Liver function tests in normal rhesus monkeys.*

| Number of monkeys | Bromsulfalein retention, percent | Number of monkeys | Thymol turbidity, units |
|---|---|---|---|
| 9 | 0 | 5 | 2.1±0.7 |
| 1 | 1.0 | 4 | 3.4±0.3 |
| 1 | 2.5 | | |
| Total 11 | Average—less than 0.5% Range—0 to 2.5% | Total 9 | Average—2.7 units Range—1.5 to 3.7 units |

The liver function tests, bromsulfalein dye excretion and thymol turbidity, were done by standard methods, using a photoelectric colorimeter. Blood was drawn 45 minutes after intravenous injection of 5 mg of bromsulfalein per kg, and the dye retained in the serum was measured. All serums were heated to 55 C for 30 minutes to destroy any viable organisms before chemical determinations were made. Control studies with unheated serums had shown that this did not interfere with the procedure in any way. Results of bromsulfalein dye excretion in 11 uninfected monkeys were similar to those reported by Evans and his colleagues[6] for normal *Macaca rhesus* and *Cynomologus* monkeys: dye retention was less than 3% in all instances and 0 in most (see table 1). Thymol turbidity in these animals ranged between 1.5 and 3.7 units. In Evans' animals it was lower—2 units or less. Bromsulfalein retention of more than 4% and thymol turbidity of more than 5.0 units were regarded as abnormal in the present studies.

Specimens of liver were obtained for biopsy using a Vim-Silverman needle. To estimate the number of viable bacteria, approximately 2 mg of liver tissue was mechanically abraded with a wire loop over the surface of a blood-agar plate, the residual tissue was transferred to a second and then a third blood-agar plate and the abrasion repeated.

Eosinophilic leukocytes of peripheral blood were stained with eosin in propylene glycol and counted in a Speirs-Levy chamber. Blood pressure was measured indirectly by placing a manometric cuff on the foreleg and auscultating or palpating the brachial artery. Hematocrit determinations were performed with a Wintrobe tube.

Roentgen examinations of the thorax were carried out in monkeys with suspected pneumonic plague.

Postmortem examination was made in all instances. Monkeys inoculated intratracheally in all instances had consolidation of one or more lobes of the lung from which *Past. pestis* was invariably cultured. In monkeys inoculated intracutaneously, cultures for *Past. pestis* of material from the ulcer at the site of inoculation were also positive.

## RESULTS

*Hepatic function in pneumonic and bubonic plague.*—Pneumonic plague was associated with early derangement of both thymol turbidity and bromsulfalein dye excretion (table 2). Thymol turbidity exceeded 5.0 units in 5 of 8 monkeys with pneumonic plague; values were abnormal in 4 on the 2nd day of fever (sustained rectal temperature of 40 C or more) and in the other on the 3rd day. Daily observation of 4 animals revealed a progressive rise of thymol turbidity units, reaching maxima of 5.5 to 10.3. Bromsulfalein retention was 6 to 26% in 7 of 8 monkeys on the 1st or 2nd day of fever. In 2 monkeys (no. 538 and 541), the bromsulfalein dye concentration reached maximal values early in the disease and diminished when the animal was moribund. This apparent improvement in hepatic function was not real, since thymol turbidity tests made simultaneously continued to yield values well above normal levels. The low value in the bromsulfalein tests under these conditions was probably attributable to the presence of large numbers of *Past. pestis* in the sample of blood.*

Hepatic function was much less deranged in bubonic plague than in pneumonic plague. Bromsulfalein excretion in 6 monkeys with bubonic plague and

6. Evans, A. S., Evans, B. K. and Sturtz, V. 1953, Standards for hepatic and hematologic tests in monkeys: observations during experiments with hepatitis and infectious mononucleosis. Proc. Soc. Exper. Biol. & Med. **82**: 437–440.

* Controlled in vitro tests of serum samples to which *Past. pestis* and bromsulfalein dye were added and the mixture was incubated at 37 C for 1½ to 2½ hours showed that the presence of large numbers of bacteria materially reduced the measurable amount of dye.

TABLE 2.—*Hepatic function in pneumonic and bubonic plague.*

| Monkey no. | Day of fever (40 C or more) | Clinical state | Thymol turbidity, units | Bromsulfalein retention, percent |
|---|---|---|---|---|
| | | Pneumonic plague | | |
| 538 | 0 | Uninfected | 3.3 | 0 |
| | 1 | Fever | 5.7 | 8.0 |
| | 2 | Fever, pneumonia, bacteremia (>5000 *Past. pestis*/ml) | 6.2 | 2.5 |
| 541 | 1 | Fever | 2.1 | 0 |
| | 2 | Fever | 5.8 | 26.0 |
| | 3 | Fever, pneumonia, bacteremia (>5000 *Past. pestis*/ml) | 6.8 | 9.0 |
| 542 | 1 | Fever | 3.7 | 0 |
| | 2 | Fever, bacteremia | 3.8 | 10.5 |
| | 3 | Fever, pneumonia, bacteremia | 5.5 | 20.0 |
| 543 | 0 | Uninfected | 2.8 | 1.0 |
| | 1 | Fever | 4.1 | 6.0 |
| | 2 | Fever, pneumonia, bacteremia | 10.3 | 8.0 |
| 527 | 0 | Uninfected | N.D. | 0 |
| | 2 | Fever, bacteremia | 3.3 | 8.7 |
| 523 | 2 | Fever | 5.1 | 7.5 |
| 533 | 2 | Fever | 2.8 | 12.0 |
| 534 | 2 | Fever | 3.5 | 2.3 |
| | | Bubonic plague | | |
| 515 | 2 | Fever | 8.0 | 0 |
| | 5 | Fever, ulcer, lymphadenopathy | 6.0 | 4.5 |
| 513 | 2 | Fever* | 4.1 | 0 |
| 514 | 2 | Fever* | N.D. | 0 |
| 516 | 2 | Fever | 2.5 | 0 |
| 500 | 4 | Fever, ulcer, lymphadenopathy | 3.1 | 3.0 |
| 507 | 4 | Fever, ulcer, lymphadenopathy | 2.1 | 11.0 |

N.D. =test not done.
* These two animals ultimately survived infection although *Past. pestis* was cultured from cutaneous ulcers; all other monkeys with bubonic plague died within 4 to 8 days of onset of fever and all those with pneumonic plague died within 3 days of onset of fever.

fever of 2 to 5 days duration was normal in all but 2 instances. In one animal (no. 515) excretion of dye was normal on the 2nd day of fever, and retention reached 4.5 mg on the 5th day. In the other (no. 507) 11% of the dye was retained on the 4th day of fever. Thymol turbidity values exceeded 5.0 units in only 1 (no. 515) of 5 animals with bubonic plague.

*Bacteriologic studies of liver in pneumonic and bubonic plague.*—Cultures of liver biopsy specimens taken from primates with pneumonic or bubonic plague yielded *Past. pestis* in the majority of the former, but in only one of the latter

TABLE 3.—*Results of culture of liver specimens from monkeys with pneumonic or bubonic plague.*

| Monkey no. | Day of disease | Day after inoculation | Liver *Past. pestis*/2 mg | Blood *Past. pestis*/0.1 ml |
|---|---|---|---|---|
| | | Pneumonic plague | | |
| 469 | 1 | 3 | 22 | 0 |
| 528 | 1½ | 4 | 250 | + |
| 527 | 1½ | 4 | 50 | 6 |
| 534 | 1½ | 3 | 115 | 0 |
| 533 | 1½ | 3 | 0 | 0 |
| 526 | 2 | 3 | 0 | 0 |
| 542 | 2 | 4 | 2 | + |
| 543 | 2 | 5 | 195 | 42 |
| 538 | 2 | 5 | 1200 | >500 |
| 541 | 2 | 4 | 0 | 0 |
| 485 | 3 | 6 | 500 | >500 |
| | | Bubonic plague | | |
| 512 | 2 | 2½ | 0 | 2 |
| 516 | 2 | 2½ | 0 | 0 |
| 500 | 3.5 | 6 | 1 | 18 |
| 507 | 3.5 | 6 | 0 | 4 |
| 513* | 5 | 5 | 0 | 0 |
| 514* | 5 | 5 | 0 | 0 |

+ =culture positive for *Past. pestis*; quantitative estimates were not made.
* These 2 animals ultimately survived infection although *Past. pestis* was cultured from cutaneous ulcers.

(table 3). In 8 of 11 monkeys with pneumonic plague there were viable bacteria in the liver; there were 2 to 1200 organisms per 2 mg of liver by the 2nd day of fever in 7 animals. *Past. pestis* was invariably cultured from the livers of monkeys with pneumonic plague and bacteremia. Moreover, on two occasions organisms were found in the liver when blood cultures were negative. In 2 animals (no. 527 and 543) the number of bacilli in 2 mg of liver exceeded that in 0.1 ml of blood. All monkeys with pneumonic plague in which bacteria were not demonstrable in the liver biopsy material were also free of bacteremia. In only 1 of 6 monkeys with bubonic plague was a liver culture positive for *Past. pestis*, and in this positive culture but a single colony grew. The animal from which this specimen was taken had bacteremia. In 2 primates with bubonic plague and slight bacteremia (no. 512 and 507) liver culture yielded no organisms.

Microscopic examination of biopsied liver tissue did not reveal any specific changes.

*Other observations in pneumonic plague.*—Daily eosinophil counts in 11 monkeys showed the persistent stress response of eosinopenia throughout the manifest disease (fig. 1). The numbers of circulating eosinophils fell sharply with the onset of fever and continued to diminish until death of the animal; at no time was there a rise towards preinfection levels in any of the animals.

Systolic blood pressure was maintained in 10 monkeys at a constant level near 110 mm Hg until less than 24 hours before death (fig. 2). Four moribund animals examined within 6 hours of death had systolic blood pressure of 60 mm Hg or more. Hemoconcentration was not evident during the course of the

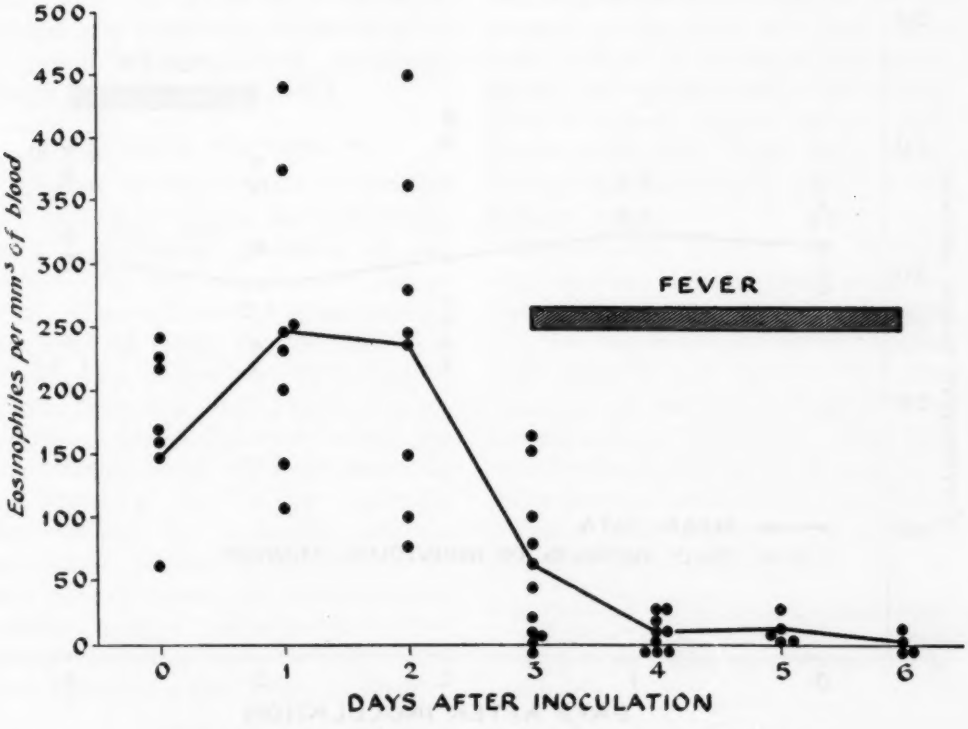


FIG. 1.—Total eosinophil count in experimental pneumonic plague.

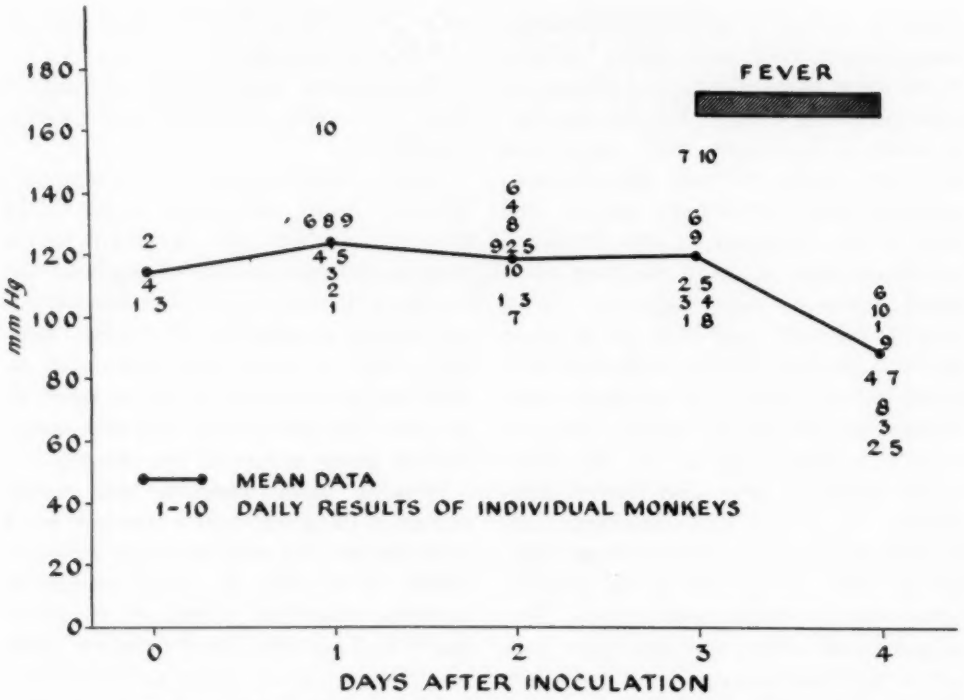


FIG. 2.—The systolic blood pressure in experimental pneumonic plague.

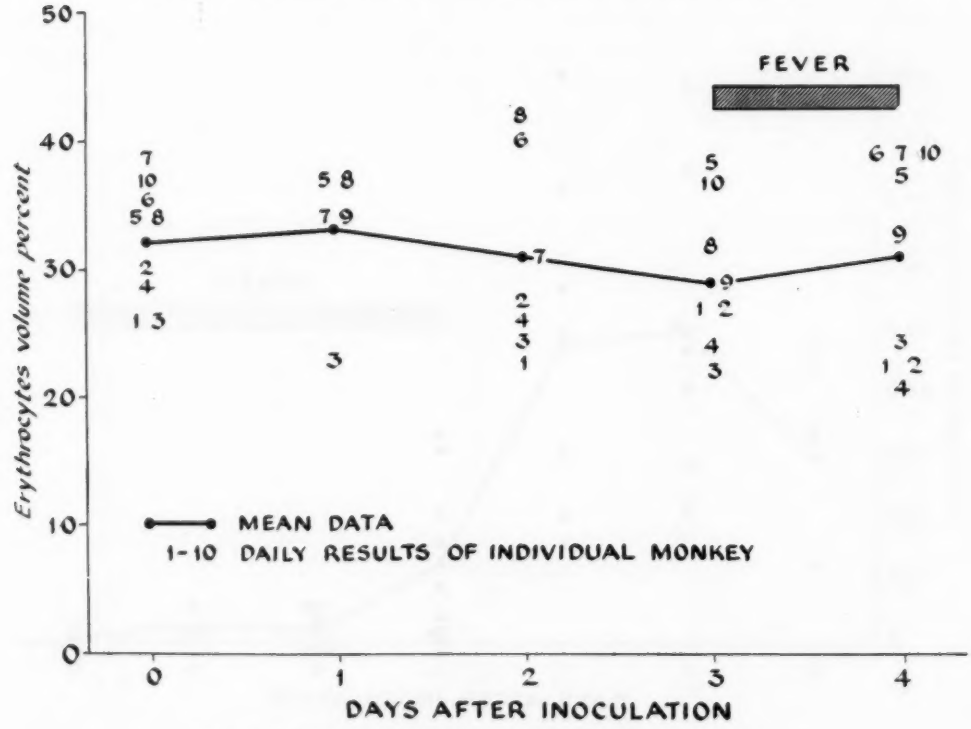


FIG. 3.—The hematocrit in experimental pneumonic plague.

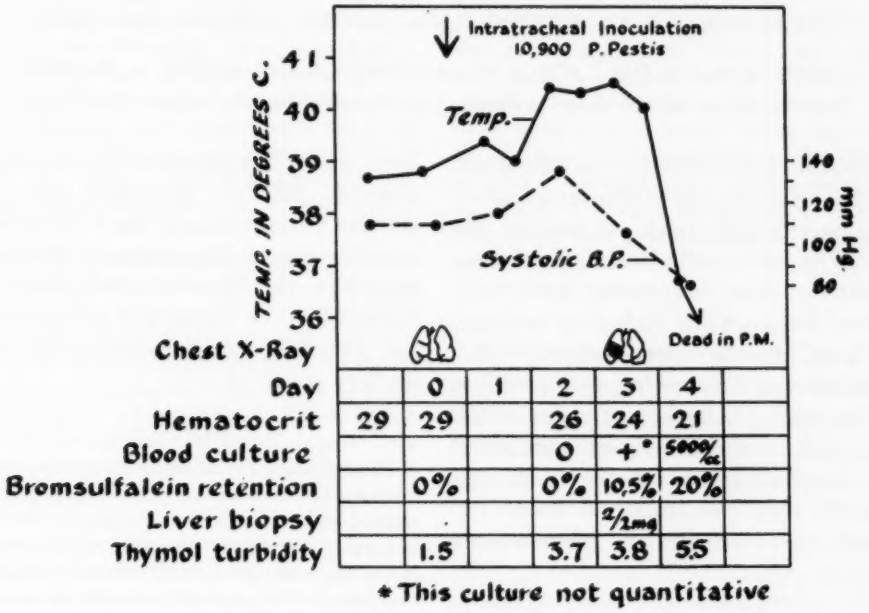


FIG. 4.—Primate pneumonic plague.

disease, even when the blood pressure had fallen (fig. 3). A daily record of clinical and laboratory observations in a typical case of primate pneumonic plague is presented in figure 4.

## DISCUSSION AND SUMMARY

Hepatic function studies of monkeys with pneumonic plague revealed diminished liver function in almost all animals by the 2nd day of fever, and in some, abnormal results coincided with the onset of fever. By contrast, in a small group of monkeys with bubonic plague there was little evidence of alteration of hepatic function through the first 4 days of fever. Whether bacteremia was present or not, the liver was regularly invaded by *Pasteurella pestis* in pneumonic plague during the 1st or 2nd day of fever. In monkeys with bubonic plague, cultures from liver biopsy specimens were virtually negative through the first 5 days of fever.

The data are limited, but it appears that in primates hepatic dysfunction and invasion of the liver by *Past. pestis* occur early in the course of pneumonic plague and before similar events take place in bubonic plague. Hepatic damage in pneumonic plague may contribute to the fulminating course of this disease.

Hypotension, save in the terminal stage of infection, and hemoconcentration were conspicuously absent throughout the course of pneumonic plague. These observations together with that of continuing eosinopenia imply that the adrenal cortex is able to maintain a hormonal response during the disease. Cortisone therapy has failed to appreciably influence the course of pneumonic plague in man or monkeys.[1,7]

7. McCrumb, F. R., Jr., Larson, A. and Meyer, K. F. 1953, The chemotherapy of experimental plague in the primate host. J. Infect. Dis. **92**: 273–287.

# SALMONELLA IN FRESH AND SMOKED PORK SAUSAGE

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Reported epidemiological studies have directed attention to edible meat products, particularly pork, as one of the sources for salmonella infections in man. Moreover, these organisms have been isolated from meats, including sausage, purchased on the open market.[1–4] Our attention was focused on this problem by the ready isolation of salmonellae from pork and beef livers purchased from local abattoirs for dog food. Apparently this contaminated meat explained the epidemic of salmonellosis then under investigation in an animal hospital. Shortly thereafter the Consultant Veterinarian of the City of Jacksonville Health Department asked that meats on the open markets under his supervision be examined for salmonellae. This was the beginning of the studies here reported.

## TECHNICS

The following technical problems received attention. The number of positive specimens increased with an increase in the amount of inoculum, up to a point. An excessive amount of meat in the tetrathionate broth inhibited its selective enrichment. The desirable amount of inoculum and the ratio of this to enrichment was determined by a series of preliminary comparative tests. In the cultures here reported a 30 g inoculum in 100 ml of tetrathionate was used.

Initially sausage meat in smaller amounts was inoculated directly to the enrichment. The fatty inoculum collected as a top layer on the broth. This made it difficult to obtain a desirable loop specimen for transfer. Moreover, it seemed apparent that the poor dispersion would markedly limit the effectiveness of the enrichment. Various materials and methods were tried to overcome this; the simplest and most effective was the addition of *d*-tergitol detergent. This was found to enhance bacterial growth within a wide range of dilutions. The amount to be added was determined by titrating to the "sudsing point," i.e., the minimum required to provide for the emulsification of the fat and to give a distinct head of foam. The quantity varied with different inoculums, but on repeated titrations it was established that 6 ml of a 10% solution of *d*-tergitol added to a 30 g pork sausage inoculum in 100 ml of enrichment was satisfactory.

The third troublesome problem was the heavy overgrowth of *Pseudomonas* and coliform organisms on plates of plain brilliant green agar inoculated from the enrichment. Extensive in vitro tests established that there was a wide difference in sensitivity to sulfonamides of these contaminating organisms and salmonella. Sulfadiazine proved more effective than sulfamylon, which was tried also. The concentration of sodium sulfadiazine, which effectively inhibited *Pseudomonas*

Received for publication February 1, 1954.

This work was done under the sponsorship of the Commission on Enteric Infections, Armed Forces Epidemiological Board, and was supported in part by the Office of the Surgeon General, Department of the Army.

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1. Cherry, W. B., Scherago, M. and Weaver, R. H. 1943, The occurrence of Salmonella in retail meat products. A. J. Hyg. **37**: 211–215.
2. Felsenfeld, O., Young, V. M. and Yoshimura, T. 1950, A survey of *Salmonella* organisms in market meat, eggs and milk. J. Am. Vet. Med. Assoc. **116**: 17–21.
3. Barsini, G. 1949, Sulla presenza de Salmonelle negli insaccati freschi di suini del commercio (Salmonella infection in fresh pork sausage exposed for sale). Riv. Ital. d'Igiene, **19**: 61–66, 1949. Abstracted in Bull. Hyg. 1951, **26**: 1100.
4. Floyd, T. M., Baranski, J. R. and El-gannani, M. 1953, Recovery of human enteric pathogens on meat from butcher shops in Cairo, Egypt. J. Intect. Dis. **92**: 224–227.

and markedly retarded the growth of coliform organisms but without detected inhibition of salmonella, was 16* mg per 100 ml of medium.

This limited study of the comparative efficacy of varying technics was not adequate to securely indicate the most effective procedures, but it did lead to the adoption of a simple routine which proved satisfactory. The technical methods employed, unless otherwise specified, were as follows:

The fresh or smoked pork sausage was purchased in the open market. The specimens were brought to the laboratory in the original one pound sealed cellophane package. Only one sample from a producer was purchased per week, and samples of all producers were collected at intervals throughout the two-year period of study. The fresh pork sausage examinations were completed first; then in a similar manner the smoked sausage samples were purchased and tested.

In obtaining the sample for culture examination, the original package was placed on a fresh paper towel and the outer cellophane was opened without touching the sausages. Sterile scissors were used to open the casings of link sausage and a 30 g sample was transferred with a sterile tongue depressor to fresh glassine weighing paper on the scale. After weighing, the sample was transferred to a 500 ml sterile ground glass stoppered bottle commonly used for the collection of bulk milk samples. Later the less expensive 16 ounce screw-capped refrigerator jars were used. To these sausage samples 6 ml of a 10% *d*-tergitol solution and 100 ml of tetrathionate enrichment broth were added aseptically. With stopper or lid in place, the bottle was shaken vigorously until the sausage sample was well dispersed. Duplicate cultures of this type were prepared from each specimen. The cultures were then incubated at 37 C for 18 to 20 hours. One loopful of each was streaked to a plate of brilliant green agar containing 16 mg of sodium sulfadiazine per 100 ml. After 20 to 24 hours suspicious colonies were picked to Kligler's iron agar with 1% sucrose added. The identification and typing procedures were those previously described.[5]

The significance of variations in culture technics warrants emphasis. Duplicate tests were done on 217 samples using the above and some other procedure. All specimens found positive were positive by the above technics. Specimens yielding few positives by the above procedures were entirely negative by other technics. In samples yielding a moderate number of positives the percentage positive was as much as 10 times higher by the technic described as compared with earlier culture methods employed. The planting of cultures in duplicate materially increased the proportion of positive specimens, since not infrequently isolations were obtained from only one of the duplicate cultures.

## FINDINGS

The number and percent of fresh and smoked sausage found to contain salmonellae are given in table 1. The sources of the product examined are divided in accordance with the probable interval between production and sale. The local abattoirs are those in or near Jacksonville. In general, their freshly prepared products are distributed promptly through the retail outlets. The local markets purchase meat in bulk and prepare sausage for immediate sale in their own shops. The regional abattoirs are those within a radius of 250 miles of Jacksonville. The remaining products tested were those from processors with a broad or nation-wide distribution. Their retail packages gave no indication of time or place of production. The added transportation time alone would account for a longer period between production and sale than for products prepared close to the distributing market. Admittedly, some samples from national distributors may have been produced within the regional area and sold promptly but there was no means of identifying such samples.

As shown in the first table, 217 samples of fresh pork sausage were examined for salmonellae and 51 (23%) were positive. The proportion positive varied according to the source of the specimen from a high of 57.5% for the products of local abattoirs to the low of 7.5% for the samples from national distributors. The number of specimens tested per pro-

* Eight mg only were found more effective in studies on poultry.

5. Galton, M. M., Scatterday, J. E. and Hardy, A. V. 1952, Salmonellosis in dogs. I. Bacteriological, epidemiological and clinical considerations. J. Infec.. Dis. 91: 1–5.

TABLE 1.—*Salmonella isolations from fresh and smoked pork sausage.*

| Source | Producer no. | Fresh sausage | | | Smoked sausage | | |
|---|---|---|---|---|---|---|---|
| | | Samples cultured | Positive | | Samples cultured | Positive | |
| | | | Number | Percent | | Number | Percent |
| Local abattoirs: | 1 | 10 | 9 | 90.0 | 11 | 3 | 27 |
| | 2 | 10 | 7 | 70.0 | 0 | | |
| | 3 | 10 | 5 | 50.0 | 0 | | |
| | 4 | 10 | 2 | 20.0 | 11 | 6 | 54 |
| | Total | 40 | 23 | 57.5 | 22 | 9 | 40.9 |
| Local markets: | 5 | 5 | 4 | 80.0 | 0 | | |
| | 6 | 3 | 2 | 66.6 | 0 | | |
| | 7 | 11 | 3 | 27.2 | 10 | 0 | |
| | 8 | 10 | 2 | 20.0 | 11 | 1 | 9 |
| | 9 | 10 | 1 | 10.0 | 0 | | |
| | 10 | 9 | 0 | | 6 | 3 | 50 |
| | Total | 48 | 12 | 25.0 | 27 | 4 | 14.4 |
| Regional abattoirs: | 11 | 5 | 3 | 60.0 | 0 | | |
| | 12 | 10 | 3 | 30.0 | 0 | | |
| | 13 | 11 | 2 | 18.1 | 8 | 0 | |
| | 14 | 13 | 2 | 15.3 | 11 | 1 | 9 |
| | 15 | 11 | 0 | | 10 | 2 | 20 |
| | Total | 50 | 10 | 20.0 | 29 | 3 | 10.3 |
| National distributors: | 16 | 9 | 2 | 22.2 | 9 | 0 | |
| | 17 | 10 | 2 | 20.0 | 0 | | |
| | 18 | 10 | 1 | 10.0 | 0 | | |
| | 19 | 10 | 1 | 10.0 | 10 | 0 | |
| | 20 | 10 | 0 | | 10 | 0 | |
| | 21 | 10 | 0 | | 0 | | |
| | 22 | 9 | 0 | | 10 | 0 | |
| | 23 | 11 | 0 | | 10 | 0 | |
| | Total | 79 | 6 | 7.5 | 49 | 0 | |
| Grand Totals | | 217 | 51 | 23.0 | 127 | 16 | 12.5 |

ducer was too small for secure opinions as to variations between the individual producers. Furthermore, the number of examinations of sausage from producers without positive findings does not warrant the opinion that their products assuredly were free of salmonellae.

Findings during the early examinations with less effective technics further indicated that sausage produced locally contained salmonellae more frequently than those from regional abattoirs or national distributors. The 20 positive samples on 92 specimens were all from local

TABLE 2.—*Types of salmonellae isolated from pork sausage.*

| Type | Total isolation | Fresh sausage | | | | Smoked sausage | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Local abattoirs | Local markets | Regional abattoirs | National distributors | Local abattoirs | Local markets | Regional abattoirs | National distributors |
| *S. derby* | 44 | 19 | 8 | 4 | 4 | 5 | 2 | 2 | 0 |
| *S. anatum* | 38 | 23 | 8 | 1 | 2 | 3 | 1 | 0 | 0 |
| *S. bredeney* | 6 | 5 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| *S. muenchen* | 5 | 2 | 0 | 3 | 0 | 0 | 0 | 0 | 0 |
| *S. newport* | 4 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 |
| *S. cholera suis* | 4 | 3 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| *S. typhi-murium* | 4 | 2 | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| *S. meleagridis* | 4 | 2 | 0 | 0 | 0 | 1 | 0 | 1 | 0 |
| *S. newington* | 2 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| *S. thomasville* | 2 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 |
| *S. montevideo* | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| *S. tallahassee* | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *S. kentucky* | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| *S. miami* | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *S. give* | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *S. atlanta* | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| *S. carrau* | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| *S. california* | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| *S. tennessee* | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 |
| Totals | 122 | 61 | 23 | 13 | 8 | 10 | 4 | 3 | 0 |

preparations, with the percentage somewhat higher for products of local abattoirs than for those of local markets. There was no positive finding during these early examinations in 10 speciments from regional abattoirs or 18 from national distributors.

There were 127 examinations of smoked sausage and 16 (12.5%) were positive for salmonellae (table 1). The proportions positive varied as for the fresh sausage. It was highest for the products from local abattoirs (40.9%), followed by those from local markets (14.4%) and regional abattoirs (10.3%). There was no positive in 49 samples of smoked sausage from national distributors.

The types of salmonellae isolated are given in table 2. This tabulation includes all salmonellae isolated from pork sausage during these studies without regard to the culture technic employed.

## COMMENT

The data on salmonellosis in hogs and cattle and salmonellae in abattoirs given in the paper immediately following contribute to the explanation of the findings recorded here. All comments are reserved, therefore, for the concluding section of the companion paper.

# SALMONELLA IN SWINE, CATTLE AND THE ENVIRONMENT OF ABATTOIRS

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The frequent recovery of salmonellae in this laboratory from pork sausage offered for sale in retail stores led to an investigation of the prevalence of salmonellosis in animals and of salmonellae in the environment of abattoirs.[1] Four commercial producers of sausage whose retail products had been examined were geographically convenient for investigation; one additional abattoir with sporadic sausage production only was studied also. Beginning in late 1950 and continuing throughout a two-year period, repeated bacteriological surveys were performed in these abattoirs.

## TECHNIQUES

All cultures were taken by cotton-tipped swabs, of medium size. Fecal specimens were obtained from the living or dead animal by rectal swab and in a small series of examinations, by perforating the seared surface of the caecum. The amount of fecal material so obtained was relatively scant from the living animal with tightly closed sphincter, whereas the post mortem rectal and caecal swabs were well coated with feces. In culturing the skin of the sides of the carcass, as was done repeatedly, the swab would be swept from ham to shoulder. Flat surfaces, as tables, were cultured by rubbing the swab back and forth about three times in an area averaging 2×8 inches. Samples from utensils, instruments and equipment were obtained by rubbing the swab over available surfaces. In the holding pens adjacent to the abattoirs, cultures were taken by swabbing the surface of cement floors, by pressing the swab into soft soil and by dipping into the water in the troughs.

The specimens thus taken were placed in tetrathionate enrichment without delay, except from plants in Jacksonville where, for convenience, the swabs were brought to the laboratory in tubes and planted about 1 hour (and never longer than 2 hours) after collection. The subsequent isolation, identification and typing techniques were those previously described.[2]

The plants were selected for study on the basis of availability. All were under the State Meat Inspection program. One plant produces quality products selling at a premium. The findings, therefore, are believed to be representative rather than exceptional.

The studies necessarily were conducted step by step throughout several months. There were no variations suggesting seasonal differences in findings. The bacteriological observations, therefore, are given in tables 1 through 4 according to material cultured and are considered in the order of progress of animals from farm to and through the processing plant.

## FINDINGS

Through the cooperation of the Florida Livestock Sanitary Board,* anal swabs were taken on live hogs on farms as the animals were being restrained for hog cholera vaccinations. In all, 374

Received for publication February 1, 1954.
This work was done under the sponsorship of the Commission on Enteric Infections, Armed Forces Epidemiological Board, and was supported in part by the Office of the Surgeon General, Department of the Army.

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1. Galton, M. M., Lowery, W. D. and Hardy, A. V. 1954, Salmonella in fresh and smoked pork sausage. **95**: 232–235.

* Now the Florida Livestock Board.

2. Galton, M. M., Scatterday, J. E. and Hardy, A. V. 1952, Salmonellosis in dogs. I. Bacteriological, epidemiological and clinical consideration. J. Infect. Dis. **19**: 1–5.

TABLE 1.—*Salmonellae in living and dead hogs found by fecal cultures taken by rectal swabs and, in one group, by caecal swabs.*

| Hogs or carcasses tested | | Examination | Positive | |
|---|---|---|---|---|
| | | | Number | Percent |
| On 28 farms—Living | | 374 | 27 | 7 |
| At abattoir —Living | | 100 | 25 | 25 |
| | —Slaughtered, same lot | 98 | 50 | 51 |
| Carcasses | —Tested by rectal swabs | 89 | 17 | 19 |
| | —Same, tested by caecal swabs | 89 | 71 | 80 |
| | —By caecal swab only | 71 | 46 | 65 |
| | —Before scalding vat | 50 | 15 | 30 |
| | —Same, after scalding vat | 50 | 9 | 18 |
| Rectal Swabs at same point in processing line | | | | |
| | Abattoir 1 | 520 | 279 | 54 |
| | Abattoir 2 | 515 | 410 | 80 |
| | Abattoir 3 | 403 | 199 | 49 |
| | Abattoir 4 | 234 | 54 | 23 |
| | Abattoir 5 | 211 | 24 | 11 |
| Total | | 1883 | 966 | 51 |

animals from 28 farms were cultured in this way and 27 (7.2%) were positive for salmonellae (table 1). However, 18 of these positives were from 34 animals on two farms where at the time of examination, the hogs were having acute diarrheal diseases. On one of these farms, 9 of 12 positives were *Salmonella miami* and on the other all were *Salmonella muenchen*. The remaining 9 positives were scattered among 6 farms. All animals tested on the other 20 farms were culturally negative. These findings do not suggest any exceptional incidence of salmonellosis among farm hogs in the southeast.

During the process of marketing, many of the hogs were taken to local sales barns. There they were purchased by a representative from an abattoir and the animals from several farms were transported together in large trucks to the meat processing plant. This mixing of animals in close quarters provided the first opportunity for the spread of salmonella infection. At the abattoirs the animals commonly were held in large holding pens for a period of 12 to 48 hours. Bacteriological findings indicated that here all hogs were heavily exposed to salmonellosis. In all, 189 specimens for culture were collected from these holding lots and 148 (78%) were positive for salmonellae (table 2). The findings on the drinking water for the hogs were particularly impressive. Sixteen samples were taken by merely dipping the swab into the water and of these, 12 (75%) yielded salmonellae. The troughs were such that the hogs could wallow in the cool water designed for drinking purposes. Furthermore, in pens without cement floors, the soil commonly proved positive for salmonellae. Comparable examinations were performed in the lots of 4 abbatoirs. In 3 of these, the propor-

TABLE 2.—*Salmonellae in the environment of abattoirs.*

| Abattoirs | Swab cultures from environment | | | | | |
|---|---|---|---|---|---|---|
| | Holding lots | | | Cutting and sausage rooms | | |
| | Cultures | Positive | | Cultures | Positive | |
| | | Number | Percent | | Number | Percent |
| 1 | 41 | 37 | 90 | 243 | 95 | 39 |
| 2 | 67 | 57 | 85 | 150 | 99 | 66 |
| 3 | | | | 193 | 85 | 44 |
| 4 | 23 | 11 | 50 | 152 | 26 | 17 |
| 5 | 58 | 43 | 74 | 111 | 1 | 1 |
| Total | 189 | 148 | 78 | 849 | 306 | 36 |

tion of positives varied from 74 to 90%. From the 4th, only 23 specimens were collected, and 17 of these with only 24% positive were obtained during a hot and dry period when the soil was "powdery dry"; in the other sampling, of 6 specimens all were positive. Thus, all hogs during the last hours or days of life were heavily exposed to salmonella infections and susceptible animals would reach the killing floor during the early or late phases of the incubation period of salmonellosis.

Cultures were taken in the holding pens on 100 live hogs and 25 (25%) were positive for salmonellae (table 1). It was impracticable to follow the same hogs to the killing floor for re-examination. However, on the same days approximately the same number of animals from the same lots were examined on the killing floor. Here, of 98 tested, 50 (51%) were found positive. The higher prevalence of salmonellosis discovered by post mortem swabs may indicate only the greater reliability of cultures with a generous fecal inoculum as compared with the pre-mortem examinations with a scant inoculum.

There was another test of the prevalence of salmonellosis in the slaughtered hogs. This was performed in an abattoir in which the proportion of animals positive by rectal swab was lower than average. From 89 animals, both rectal and caecal swabs were collected. By the former technique, 17 (19%) were found positive; by the latter, 71 (80%) yielded salmonellae. An additional 71 caecal swabs were taken from animals at two other abattoirs not examined by rectal swab, and of these, 46 (65%) were positive for salmonellae. These data suggest that at the time of slaughter the animals actually were in an early incubation phase of salmonellosis with the infection still concentrated in the lower small intestine and caecal areas.

Post mortem rectal swabs taken at a uniform location "in the line" immediately prior to evisceration and by a uniform technique were used to measure variations in prevalence of salmonellosis in hogs processed in the 5 abattoirs studied. These data also are given in table 1. (The abattoir numbers correspond with those used in the preceding report on salmonellae in sausage.) In general, the proportion of positive findings varied with the size and activity of the abattoir. The two small plants (4 and 5) handling few animals or killing at intervals only had a low proportion of positives. The others operating continuously with heavy volume had a high proportion of positives. In all, 1883 post mortem rectal swab cultures were taken and 966 (51%) were positive for salmonellae.

After leaving the killing station, the hogs are dropped into the scalding vat. Cultures of vat water were all negative for salmonellae. Likewise, the swab cultures of the sides of the hogs taken as the carcass was being lifted from the scalding vat were negative.

The carcass passed next through the dehairing machine. Here, a series of mechanical paddles scrape the skin as the carcass is rotated with a tossing action rather than by a gentle rolling movement. The carcass is subjected to vigorous treatment, and in the process, fecal leakage from the completely relaxed anus inevitably will occur. The moving parts of the dehairing machine are rather inaccessible, hence difficult to clean. These factors favor the dissemination of pollution during this stage of processing and this step of the procedure has been examined with particular care. The first column of table 3 records the result of bacteriological examinations of the dehairing machine. Of 254 cultures, 104 (41%) were positive. The proportion

positive was 20% in a small abattoir[4] and was 4% in a large one[3] in which the dehairing machine was continuously exposed to jets of water at high temperature. The percentage positive was 54 and 68 in the other two large abattoirs.

In one abattoir the effect of added attention to the cleaning of the dehairing machine was measured by daily swab cultures of this equipment, with findings as summarized in table 4. Rather casual cleaning as was done at first reduced the percentage of positive cultures from 69 to 52. With a carefully supervised and exacting cleaning the positive cultures dropped to 10%. The proportion of positive findings increased rapidly to 40% in the week immediately following the termination of this supervised cleaning.

Swab cultures of the sides of hogs were taken immediately after removal from the dehairing machine, again at the end of the evisceration line and a third time after overnight or longer storage in the chill room. With rare exceptions the same animals were swabbed at the beginning of the processing line and at its end after evisceration. Concurrent rectal swabs also were obtained from these carcasses. Cultures were taken in the chill room on the same day and were from the animals killed in the same abattoirs on the preceding day or earlier. Comparative findings on the swab cultures of the dehairing machine and the sides of the carcass tested at varying times are given in table 3. The data in table 4 are of similar nature, though without the examinations in the chill room. In considering these findings, it is to be remembered that cultures of the scalding vat water and of the sides of animals after scalding before going through the dehairing machine were culturally negative for salmonellae. However, immediately after the dehairing process a high percentage of the cultures were positive for salmonellae. It was apparent that the skins of many animals were inoculated with salmonellae as they passed through the dehairing machine.

The proportion of animals with skin cultures positive for salmonellae varied with the degree of pollution of the dehairing machine with these organisms. There were few positives from the dehairing machine in abattoir 4 (table 3) or after exacting cleansing (table 4), and corresponding skin cultures had few positives also. Cultures of the dehairing machine in abattoir 3 which was exposed to a continuous hot water spray were rarely positive, but the sterilizing procedure under trial was not highly effective in reducing the skin contamination. In general, when cultures of the dehairing machine were highly positive, so were the swab cultures of the skin.

Each carcass after dehairing passes through a triple cleansing process. It is singed with a gasoline blow torch, exposed to a jet of live steam and is washed down with a spray of cold water. Evisceration follows. Tables 3 and 4 compare the degree of skin contamination at the beginning and end of this phase of processing. In general, if there is heavy skin contamination after dehairing, the cleansing process tends to reduce this. However, one group with little skin contamination after leaving the dehairing machine had more after evisceration. The latter suggests that some skin contamination may be acquired during the evisceration process.

The limited number of skin cultures

3. Williams Smith, H. 1952, The evaluation of culture media for the isolation of *Salmonellae* from feces. J. Hyg. **50**: 21–36.

4. Hauser, G. H., Treuting, W. L. and Breiffelh, L. A. 1945, An outbreak of food poisoning due to a new etiological agent—*Salmonella berta*. Pub. Health Rep. **60**: 1138–1142.

TABLE 3.—*Salmonella isolations from dehairing machine, and the apparently related skin contamination with salmonellae.*

| Abattoir | Materials cultured | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Dehairing machine | | | Sides of carcass after dehairing | | | Sides of carcass after evisceration | | | Sides of carcass in chill room | | |
| | Number examination | Positive | | Number examination | Positive | | Number examination | Positive | | Number examination | Positive | |
| | | Number | Percent | | Number | Percent | | Number | Percent | | Number | Percent |
| 1 | 123 | 67 | 54 | 172 | 105 | 61 | 175 | 87 | 50 | 45 | 21 | 47 |
| 2 | 41 | 28 | 68 | 60 | 39 | 65 | 60 | 30 | 50 | 56 | 10 | 18 |
| 3 | 55 | 2 | 4* | 115 | 34 | 30 | 114 | 23 | 20 | | | |
| 4 | 35 | 7 | 20 | 62 | 9 | 15 | 60 | 4 | 7 | 31 | 5 | 16 |
| Totals | 254 | 104 | 41 | 409 | 187 | 46 | 409 | 144 | 35 | 132 | 36 | 27 |

* Dehairing machine subjected to a continuous spray of hot water.

on carcasses in the chill room indicate some decline in positive findings with time. The rate or degree of change can be ascertained dependably only through identical examinations on the same carcasses at known intervals.

Considering the remaining processes on the killing floor, after the triple cleaning, the animal is eviscerated and the head removed. Only 23 cultures were taken from tables and containers in this area; 17 (74%) were positive for salmonellae. The remaining steps in processing were carried on in the cutting and the sausage rooms. In the former, those portions of the carcass as the hams, loins, etc. to be sold intact were separated and prepared for wrapping. Here also the meats to be used for the preparation of sausage were collected. In the sausage room the meat was ground, mixed, seasoned and casings were filled. More than 800 swab cultures were taken in these two areas and in all, 36% were positive for salmonellae (table 2). The proportion of positive findings on the tables, receptacles, knives and mechanical equipment varied only from 25 to 43%. From these areas, and from the meat itself, salmonellae were isolated with surprising ease. The proportion of positives in the sausage room was a little higher than that in the cutting room.

The cultures obtained in abattoirs were taken under normal operating conditions. We were free to obtain any specimens desired provided there was no stoppage in the processing line. This environment was not favorable for a careful examination of the relative importance of endogenous and exogenous sources of salmonellae found in edible meats. An attempt was made to secure suitable tissue specimens free of exogenous contamination. Routinely each specimen was examined for coliform organisms as well as for salmonellae. The first specimens collected were found to have heavy coliform contamination, and the salmonellae isolated were presumed to

TABLE 4.—*The effect of extra cleansing of dehairing machine on the occurrence of contamination with salmonellae in abattoir no. 1.*

| Condition of dehairing machine when cultures taken | Materials Cultured | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Dehairing machine | | | Sides of carcass after dehairing | | | Sides of carcass after evisceration | | |
| | Number examination | Number | Percent | Number examination | Number | Percent | Number examination | Number | Percent |
| Before extra cleaning | 13 | 9 | 69 | 22 | 20 | 91 | 24 | 18 | 75 |
| Some extra but still inadequate cleaning | 25 | 13 | 52 | 43 | 41 | 95 | 44 | 25 | 57 |
| Exacting cleansing | 20 | 2 | 10 | 40 | 1 | 3 | 40 | 7 | 18 |
| After termination of supervision of cleaning | 20 | 8 | 40 | 25 | 22 | 88 | 25 | 21 | 84 |

be the result of fecal pollution. However twenty carcasses were examined under better conditions, permitting the collection from each of specimens of mesenteric and portal lymph glands, sections of liver and spleen and portions of muscle tissue. The external surface of each specimen was cleansed by dipping in alcohol and flaming before the centrally located tissue was separated, minced and cultured. With this procedure, no coliform contamination was found. Among 100 cultures of tissues, only one, a mesenteric lymph gland, was positive for salmonella. That specimen came from a hog whose fecal culture was positive for *Salmonella derby;* the lymph gland yielded the same type of salmonella.

Findings believed significant were obtained from an earlier and separate series of 125 tissue specimens obtained from 25 animals before the coliform contamination was successfully eliminated. In this series taken on one day in a small abattoir, 23 (18%) of the 125 tissue cultures yielded *Salmonella cholerae suis*. The positive specimens came from 10 hogs and in two of these each of the 5 specimens collected were positive. Organisms were found in 7 of the 25 liver specimens, in 6 spleens, 4 muscle specimens and three times each from mesenteric and portal lymph glands. The findings were similar to post mortem tissue cultures collected on two hogs which died with symptoms of hog cholera, where 9 of 10 tissue specimens yielded *S. cholerae suis*. Seemingly well hogs from sick herds may be encountered occasionally as a group in small abattoirs and here systemic invasion with salmonellae may be an important factor.

### *Related studies of cattle*

Cattle on dairy farms were accessible and easy to handle. Fecal cultures were obtained by rectal swabs from 1196 animals from 7 dairies. The 596 cultures taken from 5 herds were all negative. There were 2 positives (*Salmonella give* and *Salmonella bareilly*) in one herd of 50 cows. In a large divided herd of 550 animals including cows, heifers and calves, 14 were positive for salmonellae (2 *S. give*, 2 *Salmonella anatum*, and 10 *Salmonella kentucky*). Here in one group of 26 calves, 8 (31%) were positive. The other 4 isolations were from 1 calf, 2 heifers and 1 cow. The latter remained persistently positive for *S. give* during the 5 months she was followed bacteriologically.

In abattoirs, all of which slaughtered cattle as well as hogs, an additional 147 fecal cultures were obtained from cattle immediately after slaughter and 17 (12%) were positive for salmonellae.

There was no opportunity to study the bacteriological environment of abattoirs handling cattle only.

### *Distribution of Salmonella types*

The 2566 salmonella cultures isolated during these studies represent 38 serological types, including one new type and two not previously encountered in Florida. The frequency of occurrence of these types in the intestinal tract of swine and the environment of the abattoirs is shown in table 5. *S. anatum* and *S. derby* were the most common types from all sources in each of the 5 plants studied. The variation in the prevalence of some types may be explained in part by a wide dissemination limited in time. For example, 51 of the 61 *Salmonella san diego* cultures were isolated from anal swabs or from the sides of the same hogs in a single survey in one abattoir, and the 16 cultures of *Salmonella worthington* were obtained only once during another survey in this plant. Similarly, the majority of the *Salmonella montevideo* and *california* cultures were isolated from 2 plants during single surveys. *Salmonella meleagridis* was found simultaneously in the holding lots, in the hogs

TABLE 5.—*Distribution of salmonella types in swine and the environment of abattoirs.*

| | Type | Fecal* cultures | Contamination on carcasses† | Holding lots | Contamination in abattoirs | Dehairing machine | Total |
|---|---|---|---|---|---|---|---|
| B | 1. *S. paratyphi B* | 1 | | | | | 1 |
| | 2. *S. saint paul* | 15 | | | 2 | | 17 |
| | 3. *S. san diego* | 25 | 35 | 1 | | | 61 |
| | 4. *S. derby* | 539 | 138 | 40 | 87 | 40 | 844 |
| | 5. *S. california* | 20 | | | 1 | 3 | 24 |
| | 6. *S. typhimurium* | 38 | 3 | 8 | 5 | 2 | 56 |
| | 7. *S. bredeney* | 52 | 8 | 1 | 12 | 4 | 77 |
| $C_1$ | 8. *S. cholerae suis* | 6 | 2 | 2 | 1 | | 11 |
| | 9. *S. montevideo* | 14 | | 14 | 2 | | 30 |
| | 10. *S. oranienburg* | 11 | 1 | 3 | 8 | 1 | 24 |
| | 11. *S. bareilly* | 1 | 2 | 1 | | | 4 |
| | 12. *S. hartford* | 6 | | | 1 | | 7 |
| | 13. *S. tennessee* | 8 | 4 | | 2 | 1 | 15 |
| $C_2$ | 14. *S. muenchen* | 14 | 6 | 5 | 1 | 1 | 27 |
| | 15. *S. manhattan* | 5 | | | 1 | | 6 |
| | 16. *S. newport* | 47 | 10 | 5 | 9 | 4 | 75 |
| | 17. *S. litchfield* | | | | 1 | 3 | 4 |
| | 18. *S. kentucky* | 5 | | | | | 5 |
| D | 19. *S. miami* | 21 | 2 | 3 | 5 | | 31 |
| | 20. *S. berta* | 3 | 2 | | | | 5 |
| | 21. *S. pensacola* | 2 | | | | | 2 |
| | 22. *S. javiana* | 10 | | | | 1 | 11 |
| $E_1$ | 23. *S. anatum* | 547 | 170 | 101 | 181 | 60 | 1059 |
| | 24. *S. meleagridis* | 22 | 10 | 3 | 5 | 5 | 45 |
| | 25. *S. give* | 30 | 17 | 1 | 10 | 1 | 59 |
| | 26. *S. lexington* | 1 | | | | | 1 |
| $E_2$ | 27. *S. newington* | 9 | 2 | 1 | 1 | | 13 |
| | 28. *S. thomasville* | 8 | | | | | 8 |
| $E_3$ | 29. *S. senftenberg* | 2 | 1 | | 3 | | 6 |
| F | 30. *S. luciana* | 2 | | | | | 2 |
| | 31. *S. rubislaw* | 3 | 2 | 1 | | | 6 |
| G | 32. *S. mississippi* | 2 | | | | | 2 |
| | 33. *S. worthington* | 2 | 9 | | | 5 | 16 |
| H | 34. *S. florida* | 3 | 1 | | | | 4 |
| | 35. *S. madelia* | 1 | | | 2 | | 3 |
| FG | 36. *S. minnesota* | 1 | | 1 | | 1 | 3 |
| | 37. *S. alachua* | | | 1 | | | 1 |
| | 38. *S. duval* | | 1 | | | | 1 |
| | Total isolations | 1476 | 426 | 192 | 340 | 132 | 2566 |
| | Total specimens positive | 1239 | 372 | 160 | 306 | 107 | 2184 |
| | Total specimens examined | 2822 | 999 | 205 | 879 | 254 | 5159 |

* Anal swabs from living and slaughtered hogs.
† Swabs from sides of carcasses.

before cleaning, on the dehairing machine, and on the hog carcasses in the chill room. Approximately 1 week later, it was isolated from a pound package of sausage produced at this plant and purchased in a local retail outlet.

Multiple salmonella types were encountered frequently, as evidenced by the 2566 isolations from 2184 positive specimens. There were 330 specimens in which 2 types were identified, 23 with 3, and 2 with 4 types. The isolation of multiple types from the same specimen was favored by the performance of duplicate cultures in studying the comparative efficacy of different mediums. Thus, frequently, from 4 to 8 colonies per sample were studied.

Of the 12 most common types found in swine and the environment of abattoirs, 9 of these are among the 12 most commonly found in man in Florida, namely, *Salmonella anatum, derby, bredeney, newport, typhimurium, miami, montevideo, muenchen* and *oranienburg*. Of the 38 types encountered 34 have been isolated from man in Florida. Three of the four others had single isolations from swine. There were only 11 isolations of *S. cholerae suis*. Williams

Smith[3] has observed that this organism was recovered from tetrathionate enrichment broth only after a heavy inoculum of organisms, whereas it was cultured readily by streaking directly to brilliant green agar. Our culture techniques, though highly sensitive for most salmonellae, apparently were relatively ineffective for the isolation of *S. cholerae suis*.

## COMMENT

The proportion of hogs in abattoirs found positive for salmonellae by rectal swab cultures was unexpectedly high. The possibility of some of the skin contamination from the dehairing machine draining into the relaxed anus when the animal was hung head down was considered. Comparative caecal swabs therefore were collected through a seared sterile surface. These yielded a markedly higher proportion of positives than the rectal swabs from the same animals. Furthermore, a series of 50 post mortem rectal swabs were taken on the killing platform for comparison with tests taken routinely "on the line." The former yielded more positives than the latter. This suggested that the findings may include "false negatives" rather than "false positives." To limit any hazard of extraneous contamination gaining entrance to cultures, single swabs were sterilized in five inch tubes. The swab was removed on taking the culture and immediately reinserted into the cotton plugged tube. The enrichment was inoculated in office quarters presumably free of salmonella contamination. Our findings so obtained and confirmed by the above comparative tests have forced us to conclude that a high proportion of hogs reaching the killing floor in the abattoirs examined harbor salmonellae in the enteric tract. It is of minor importance practically whether the true proportion of hogs infected with salmonellae at the time of slaughter is 25, 50 or virtually 100%. The evidence calling for attention is that there are numerous sources for the spread of salmonellae to edible meat products.

Evidence indicates that there is a wide difference in the proportion of infected hogs on the farm and in the abattoir. The numerous and congested contacts with possibly infected hogs or with salmonellae in the environment in sales barns, during transportation and in holding lots all favor the spread of salmonellosis. The bacteriological environment of the holding lots in which the hogs are kept immediately prior to slaughter would account for a high prevalence of infection in slaughtered hogs. Apparently many may be killed during the incubation stage of subclinical or clinical salmonellosis. Each infected animal is a possible source for the spread of salmonellae in the abattoir. Thus the major cause of the problem of salmonellae in meat products is the spread of salmonellosis during the hours and days immediately prior to slaughter.

Within the abattoirs observed, the dehairing machines served to inoculate the skin of the carcasses with salmonellae. The evidence of skin contamination with these organisms was reduced only moderately by the subsequent cleaning process and by storage in the chill room. Thus, operations in evisceration and cutting of the carcasses would be performed through a contaminated field.

In the present stage of knowledge one can only speculate as to the multiplication of salmonellae in the environment of the abattoir. Blood, serum, and tissue fluids suitable for the cultivation of salmonellae were readily available. Cultures of equipment (as the dehairing machine) prior to the beginning of the day's work yielded as many positives as during operation. Certainly there ap-

peared to be many foci in the abattoirs where salmonellae could multiply.

Blood stream dissemination of salmonellae to the tissues apparently occurs occasionally. However, it was clearly evident that there was a ready distribution of fecal pollution within the abattoirs, and the findings suggested that the salmonellae in edible meat is the result primarily of the spread of pollution during processing.

There was a marked variation in the occurrence of salmonellae in sausage produced in local abattoirs and markets, as compared with products from regional abattoirs and producers with nationwide distribution. The explanation of this is not evident. It may be the difference between very fresh products and those stored for varying lengths of time. However, there may be true regional variations related to differences in the incidence of infection in hogs, variations in the nature or control of the processing procedures employed, or to climatic factors affecting the viability or multiplication of salmonellae in the environment of abattoirs. There may be other unsuspected factors. Certainly comparative studies in representative areas and abattoirs are indicated. This will require the unreserved and friendly cooperation of the industry similar to that so generously given by the local plants studied.

There is in Florida a close similarity in types of salmonellae found in man and swine. Of the 12 most common types found in man, 9 were among the 12 most common types in swine. This similarity suggests either common sources or dissemination from the animal to human host (or, less likely, spread in the opposite direction). Studies by others have pointed the finger of suspicion to pork. Hauser and associates[4] recovered *Salmonella berta* from pork sausage and from 6 ill persons examined bacteriologically. Similarly, Jones and Symons[5] isolated *Salmonella dublin* from human cases and from remaining portions of suspected sausage. Petzel[6] studied an outbreak involving 153 persons. *Salmonella typhimurium* was isolated from both the sausage and the cases. Other meats have been incriminated even more frequently. British investigators[7] summarize as follows their opinions as to the source of 2021 food poisoning incidents occurring in 1950: "Processed and made-up meat dishes are by far the most important foods in the spread of infection. Forty-eight per cent of all outbreaks where the vehicle of infection was ascertained were associated with meat pies, sausage meat, brawn, cold meats, stews, reheated and made-up dishes, pressed beef, rissoles, gravy, stock, and similar dishes." The importance of pork products, including sausage, as a possible source of salmonellosis in man warrants emphasis.

The knowledge that potential pathogens are commonly present in edible meats on the open market is disquieting. Furthermore, the evidence that this is predominantly an indication of fecal pollution is disturbing. Public health officials have given commendable attention to the cleanliness and bacterial qualities of dairy products with gratifying results. It is scarcely reasonable that so little public health attention is given to the cleanliness and bacterial qualities of edible meats. We are impressed with the possible value of cultures for sal-

5. Jones, A. C. and Symons, A. D. 1948, An outbreak of food poisoning due to *S. dublin* conveyed by sausages and sausage meat. Monthly Bull. Min. Health & Emer. Pub. Health Lab. Ser. **7**: 202–206.
6. Petzel, C. 1949, An outbreak of meat food poisoning in a hospital. Ztschr. f. Hyg. u. Infektionskr. Abstracted in Bull. Hyg. 1949, **24**: 889–890.
7. Cockburn, W. C. and Simpson, E. 1951, Food poisoning in England and Wales, 1950. Monthly Bull. Min. Health & Pub. Health Lab. Ser. **10**: 228–239.

monellae of the environment of abattoirs and of meats as a temporary index of unwarranted pollution. Later, in meats, as in milk, it may be practicable to use a coliform index as a measure of degree of fecal pollution.

The meat processing industry clearly has sanitary problems difficult to control. This begins with the tendency of some livestock producers to dispose of known infected herds or of mildly ill animals while they still can be sold. There is no practical means of detecting animals with a subclinical salmonella infection among those offered for sale. Moreover, there is an unavoidable congestion during marketing, transportation and holding at the abattoirs which favors a pre-mortem spread of infection. Within the abattoirs the nature of the operation does not favor cleanliness, and many of the employees who are willing to work in abattoirs are undersensitive as to its importance. Possibly the problems observed are effectively controlled by some abattoirs, but if so, their methods should be applied more widely.

The basic need is for a proper concern for the bacteriological environment of abattoirs and the bacteriological quality of meat products. Some of the accepted methods in the public health control of the cleanliness, sanitation and safety of dairy products could with advantage be adapted for the progressive improvement of the sanitary quality of meat products available to the American public.

# SOME METABOLIC ASPECTS OF HOST-PARASITE INTERACTION USING THE ALBINO MOUSE AND *SALMONELLA TYPHIMURIUM*

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Survival time of mice artificially infected by intraperitoneal injection of a saline suspension of *Salmonella typhimurium* is reduced from an average of more than 72 hours to less than 24 hours by sublethal quantities of certain inhibitors and intermediates of the tricarboxylic acid cycle (Berry and Mitchell, 1953a, b, c). Malonate is the most effective of ten compounds tested (the results with only five have been reported). This is not the case with all of the six additional pathogens which have been studied (Berry, Merritt and Mitchell, 1954). There seems to be some degree of specificity between the in vivo effects produced by the different substances and the parasite's ability to "benefit" from them. Moreover, it has been observed that when the number of bacteria introduced is incapable of killing control mice, 100% mortality may result when repeated injections of sublethal quantities of the appropriate compound are given. The injection of substances potentially capable of altering reactions in the citric acid cycle may, under certain conditions, therefore, convert an otherwise sublethal infection into one that is fatal for an entire group of animals.

These observations open a new avenue of attack on the biochemistry of host-parasite interaction. While it is obvious that the parasite must derive its complete nutriment from the host, few of the details of the mechanisms by means of which this is accomplished are known. In the case of bacterial infections, at least two phenomena must occur concomitantly. In the first place, the microorganisms must increase in number within the host, competing in some way for the necessary compounds they require for growth and multiplication. Then, secondly, as the parasite population grows larger, the toxic manifestations of the infection must become progressively more severe.

The number of bacteria appearing in the blood stream of artificially infected mice following malonate injections is qualitatively larger than the number in the blood of mice injected with an equal volume of saline (Berry and Mitchell, 1953b). Under the same conditions, the survival time is reduced. It becomes important then to know more exactly the rate at which the bacteria increase in mice under the same experimental conditions that lead to reduced survival time. The first part of this communication is concerned with this problem. A preliminary report has been submitted elsewhere for publication (Berry and Mitchell, 1954).

The toxic manifestations of pathogenic bacteria are thought to be due to the action of exotoxins, endotoxins or both. Injections of some of the former may produce symptoms characteristic of that particular toxin, while adminis-

Received for publication February 1, 1954.

This study was supported at Bryn Mawr College by funds provided under Contract AF 18(600)-551 with the USAF School of Aviation Medicine, Randolph Field, Texas.

* With the technical assistance of Marylin P. Hill and Beverly S. Morris.

tration of the latter usually results in nonspecific effects. Experimental animals may die within a few hours following the introduction of a sufficient number of heat-killed bacteria, but they may survive if smaller numbers are involved. It was recently reported (Berry, Ehlers and Mitchell, 1954) that the injection of *S. typhimurium* endotoxin into mice followed, after approximately 15 hours, by injections of sublethal quantities of malonate, results in the death of all mice within 1 to 4 hours. None of the animals receiving the same quantity of endotoxin and the same number of injections of saline without malonate died within this period of time; however, 2 of 9 mice succumbed the following day. Toxic amounts of endotoxin were employed, but the cause of rapid death in the animals given malonate must be sought in terms of a summation of biochemical effects. The second part of this report is concerned with the results of studies designed to elucidate this aspect of the problem.

## METHODS

*Determination of the number of* S. typhimurium *in the blood of mice.*—The number of bacteria in the blood of mice artificially infected with *S. typhimurium* was determined by the method described previously (Berry and Mitchell, 1954). Briefly, this involves amputating the end of the mouse's tail, drawing the blood to the "1.0" mark of a calibrated small red blood cell diluting pipette, diluting the blood with heparinized saline, and, after thorough shaking, discharging the full contents of the pipette onto the surface of a Petri dish containing SS agar. The number of colonies developing, times the ratio of 1 cc. to the absolute volume of blood used (this is 1000 for pipettes with a volume of 0.001 cc), gives the estimated number of bacteria per cc of blood. Under experimental conditions shown by preliminary test to yield bacterial numbers in excess of those reliably counted by this procedure, additional dilution is required. Accordingly, the contents of the red cell pipette are discharged onto a clean glass slide and a calibrated white blood cell diluting pipette is filled to the "1.0" mark with this diluted blood, and then to the "11" mark with saline. After shaking, the contents are spread over the surface of SS agar. With the particular pipettes employed, the number of colonies times 11,000 gave the estimated number of bacteria per cc of blood.

For each individual experiment, mice in groups of 10 were infected intraperitoneally with 0.5 ml of a standardized suspension of *S. typhimurium* containing about 250,000 bacteria, the preparation of which has been described previously (Berry and Mitchell, 1953b). Immediately thereafter, half of the mice were injected with 20 mg sodium malonate (Eastman) dissolved in 0.5 ml of sterile 0.9% sodium chloride solution, and the remaining half were injected with the same volume of saline alone. The injections were repeated at hourly intervals until a total of 8 injections had been administered. Beginning 1 hour postinfection, blood samples were taken from each of the 10 mice in turn. When all mice had been bled, the procedure was repeated until each animal had been sampled about 5 times within a 12-hour period. Survivors were sampled again on succeeding days. Records were kept for the individual mice and the time of death was recorded to the nearest hour postinfection. The mice were marked with a color code which made possible identification of the individual animals.

In addition to malonate, injections of fluoroacetate, citrate, and succinate were given infected mice in amounts and at intervals used previously (Berry and Mitchell, 1953c). They received two injections of sodium monofluoroacetate (kindly supplied by Monsanto Chemical Co.), the second following the first by 6 hours. Each injection contained 8 mg per kg dissolved in approximately 0.5 ml of saline. Injections of citrate and succinate were given, like malonate, at hourly intervals for a total of 8 injections Each injection of citrate (trisodium, Merck) contained 10 mg in 0.5 ml of saline and of succinate (disodium, Merck) contained 20 mg in 0.5 ml of saline. Bacterial counts on the blood of mice so treated were made so that they might be compared with those for mice given malonate.

*Determination of blood sugar in mice.*—Blood glucose was determined by the method of Benedict (1931). A Coleman model 11 spectrophotometer was used for measuring the color.

Volumes of blood ranging from 0.25 to 0.5 ml were obtained by decapitation. The blood was collected in a paraffined watch glass and its volume was measured in a tuberculin syringe. It was placed in a 50 cc Ehrlenmeyer flask which contained 3.0 ml of distilled water. The flask was swirled vigorously each time the blood of from 2 to 5 mice was added. When the pooled sample had been collected, enough distilled water was

measured into the flask to bring the final volume to 7 times the total volume of blood. After the addition of one blood volume of 10% sodium tungstate solution, an equal amount of 2/3 *N* sulfuric acid was added drop by drop from a buret while the flask was constantly shaken. After 5 minutes the precipitated blood was filtered through qualitative filter paper and the filtrate was collected in a 50 cc beaker. The Benedict test for glucose was carried out on this filtrate.

*Preparation of* S. typhimurium *antigen.*—*S. typhimurium* antigen (or endotoxin) was prepared by inoculating 10 ml of brain heart infusion broth (Difco) from a stock culture and incubating the tubes for 19 hours at 37 C. The number of bacteria contained in such a culture, as determined by dilution counts, is about 2 billion cells per ml. The culture was heated for 1 hour in a water bath held between 60 to 70 C. Subcultures into nutrient broth were made to insure that no viable organisms remained. The antigen was stored in the refrigerator. Each mouse was injected with 0.5 ml of this preparation. As a check on this type of antigen, another was prepared by growing the bacteria on nutrient agar slants, washing them free with a stream of sterile saline, centrifuging and discarding the supernatant and repeating this three times in order to free the cells of foreign substances that might dissolve out of the nutrient material. The final suspension of cells was heat-killed as described above immediately after a dilution count had been made in order to determine the cellular density of the suspension. The final preparation was adjusted with saline until there were about 2 billion cells per ml.

*Lactic acid determination in organs of mice.*—Lactic acid was assayed, according to the method of Umbreit et al (1945), in blood, duodenum, heart, kidney, liver and spleen. Each determination was made on pooled samples of the individual organs of from 3 to 5 mice.

Blood, collected and measured as described above, was placed in a 100 cc Ehrlenmeyer flask. Immediately thereafter, 10 times this volume of 10% trichloroacetic acid and an equal volume of tap water were added. The flask was agitated on a Boerner shaker until the next sample of blood was added. Finally, the contents of the flask were transferred to a centrifuge tube and centrifuged. The assay was carried out on 1 ml of the supernatant and the number of micrograms it contained times 12 gave the weight of lactic acid per ml of blood.

The other tissues were dissected out as quickly as possible by one member of a team and placed directly on a block of dry ice. They were weighed in the frozen state on a torsion balance by the other member of the team and returned to the dry ice until all organs were ready to be homogenized. The tissues were then placed in homogenizing tubes, one for each type of organ, which contained 2 ml of ice cold isotonic potassium chloride solution. Following homogenization, additional potassium chloride was added to bring the final volume to 1 ml per 100 mg of tissue. Proteins were precipitated by the addition of an equal volume of 20% trichloroacetic acid and, following thorough stirring of this mixture, 1 ml was transferred to a small test tube which contained 1 ml of distilled water. This was centrifuged and the assay was carried out on 1 ml of the supernatant. The weight of lactic acid in this material times 40 gave the number of micrograms per gram wet weight of tissue.

*General.*—Insulin was injected into mice by diluting the commercial preparation (Iletin, Lilly) which contained 20 units per ml in such a way that 1 unit was present in 0.5 ml. Saline was used as the diluent. Mice were injected with 0.1 ml of 1:1000 dilution of adrenalin chloride (Parke, Davis). All injections were made intraperitoneally.

Young adult female mice of the CF 1 strain were used in these studies. All animals were housed in small cages containing wood shavings. They were fed Purina dog chow and water was available at all times.

## RESULTS

*1. Bacteremia in mice injected with inhibitors or intermediates of the tricarboxylic acid cycle.*—Before the effect of injections of inhibitors or intermediates of the tricarboxylic acid cycle on the severity of the bacteremia which develops in artificially infected mice could be experimentally determined, it was first necessary to establish the time-course of the blood stream infection in a group of control mice. Accordingly, it was found that mice infected by intraperitoneal injection of a standardized suspension of *S. typhimurium* followed by hourly injections of saline develop a bacteremia within 1 to 2 hours. A peak value is reached at 7 hours postinfection, and this is followed by a decrease in the number of bacteria per unit volume of blood which approaches zero between 12 and

24 hours. Thereafter, as the infection becomes more severe, the organisms return to the blood stream in larger and larger numbers until death intervenes. This relationship can be seen in table 1, the column designated "saline." Each value was obtained by averaging the number of bacteria per ml of blood determined experimentally at each of the different intervals postinfection in a total of 42 mice. Counts were not made at each interval on all mice. The largest number involved was 39 mice at 5 hours and the smallest number was 5 mice at 72 hours.

These data give quantitative confirmation of a relationship described by Webster (1933) about 20 years ago. He determined, by culturing the blood of mice infected intraperitoneally with *Salmonella enteritidis*, the time at which bacteria first appeared in the blood and the time at which they subsequently disappeared. He found positive cultures after 2 to 4 hours post-infection and negative cultures, as a rule, at 12 hours. With our mice, the blood is positive the first hour in about half the mice and it is still positive in about one-third the mice at 12 and 24 hours. This is in good agreement with Webster's findings, even though he used a different organism and a different method. At 48, 53, and 72 hours postinfection, the number of bacteria in the blood stream of control mice increases exponentially. The final count, at 72 hours, averaged 406,000 bacteria per ml of blood. At this time the first deaths occur and they continue for the next 4 or 5 days. The average time of death for these mice is 110.6 hours postinfection, as shown in table 2, column 5.*

To be compared with the results for control mice are those obtained for mice similarly infected but given injections of malonate, fluoroacetate, succinate, or citrate. The average number of bacteria per unit volume of blood at different times postinfection in mice given one of the different compounds are presented in table 1. The largest number of bacteria appears in the blood of animals injected with malonate. At 9, 10, and 12 hours postinfection, these mice have a mean bacterial count of more than 3 million per ml of blood. Since some animals died by the 8th hour postinfection, the peak bacterial counts should be considered characteristic of only the survivors. At 13 hours postinfection, 10 of 15 animals were dead and the remaining 5 were dead at 24 hours (see table 2). In-

* Average survival times as shown are larger than true values since animals dying overnight were listed as having died at the hour when they were found.

TABLE 1.—*Mean number of bacteria per ml of blood in different groups of mice infected intraperitoneally at time 0 with 250,000 cells of* S. typhimurium.

| Time postinfection (hours) | Mean number of bacteria per ml of blood from mice given injections of | | | | |
|---|---|---|---|---|---|
| | Saline (8×0.5 ml) | Malonate (8×20 mg) | Fluoroacetate (2×8 mg/kg) | Succinate (8×20 mg) | Citrate (8×10 mg) |
| 1– 1.5 | 2,100 | 1,300 | 4,000 | 2,200 | 2,000 |
| 2– 2.5 | 4,800 | 27,400 | 11,400 | 4,300 | 3,300 |
| 3– 3.5 | 8,300 | 46,600 | | | 3,700 |
| 4– 4.5 | 9,400 | | 100,600 | 23,400 | 24,800 |
| 5– 5.5 | 13,000 | 80,000 | 422,000 | 40,750 | 43,200 |
| 6– 6.5 | 18,100 | 344,000 | 790,800 | 111,700 | 86,800 |
| 7– 7.5 | 26,700 | 649,000 | | | 76,200 |
| 9– 9.5 | | 3,300,000 | | | |
| 10–10.5 | 16,800 | 3,600,000 | | 232,000 | 42,400 |
| 11–12 | 1,900 | 3,370,000 | 866,000 | | 5,000 |
| 24 | 1,600 | | | 392,000 | |
| 48 | 21,900 | | | | |
| 53 | 38,400 | | | | 300,000+ |
| 72 | 406,000 | | | | |

Injections of all compounds were given by the same route at hourly intervals except fluoroacetate. With this compound, the second injection was given 6 hours after the first.

TABLE 2.—*The mean maximum number of bacteria and the standard deviation of the mean, appearing in the blood stream of all mice within a group during the first 12 hours post-infection and the mean survival time ± the standard deviation of the mice in each group. The range of values for each mean and the number of mice per group are given.*

| Mice injected with | Number mice tested | Average maximum number of bacteria | Standard deviation of mean number | Range of values for maximum numbers | Average survival time ± standard deviation | Range of survival times |
|---|---|---|---|---|---|---|
| Saline (8×0.5 ml) | 42 | 31,142 | 44,180 | 2,000– 221,000 | 110.6±25.1 | 72–192 |
| Malonate (8×20 mg) | 15 | 1,796,000 | 2,411,500 | 90,000–8,300,000 | 14.8± 7.0 | 8– 24 |
| Fluoroacetate (2×8 mg/kg) | 5 | 1,281,000 | 972,000 | 465,000–2,940,000 | 21.2± 6.3 | 10– 24 |
| Succinate (8×20 mg) | 10 | 210,000 | 162,400 | 90,000– 560,000 | 50.6±26.6 | 24– 96 |
| Citrate (8×10 mg) | 17 | 162,900 | 280,900 | 18,000–1,150,000 | 70.3±20.3 | 24– 96 |

fected mice injected with fluoroacetate develop a severe bacteremia as early as mice given malonate, even though the maximum average number of bacteria in these mice never exceeds 900,000 per ml. Some of the individual mice in this group had well over a million organisms per ml of blood and none survived beyond 24 hours. The initial deaths occurred later and the average survival time was longer than in mice given malonate. This is shown in table 2.

Mice given succinate have slightly less than 400,000 bacteria per ml of blood at 24 hours postinfection. The bacteremia becomes steadily more severe up to this time when the first mortalities occur. This may be seen in tables 1 and 2. Citrate, on the other hand, produces a bacteremia just as severe as succinate during the first 5 hours (table 1), but by the end of 12 hours postinfection, the number of bacteria decreases, as it did in control mice. The average survival of mice given succinate is much longer than it is for animals injected with fluoroacetate but it is nearly 20 hours less (but not statistically significant) than it is for citrate, as shown in table 2.

Not only is the time course of the bacteremia important, but possibly more significant, in view of the individual variation in survival time of mice, is the mean maximum number of bacteria appearing in each group during the first 12 hours postinfection. The mean values and their standard deviations are presented in table 2, including the range of values for each group and the number of animals on which counts were made. Similar data are given for survival time, already referred to in the paragraphs above. A study of these data brings out the following facts: (a) The greatest average number of bacteria per ml of blood occurs in mice given malonate, followed, in order, by animals injected with fluoroactetate, succinate, citrate, and finally control mice. (The average maximum numbers are based on values for all mice, irrespective of time, and hence differ from the values in table 1.) (b) When the difference in mean maximum number of bacteria between one group and that for the other groups is tested in turn for significance by the Mann-Whitney test according to the method of White (1952), a probability of less than 0.1% is found for each experimental group compared to the control group. The differences between non-control groups are all significant at the 1.0% level except in the cases of malonate vs. fluoroacetate and succinate vs. citrate. The two exceptions were not significant even at the 5% level. (c) The significance of the difference between means exists despite the very large standard deviations which reflect large

variations in individual values within a single group of mice. (d) Mean survival times are inversely related to the mean maximum number of bacteria. (e) Statistical analysis by the method of White (1952) of the differences in mean survival time between all groups gave a probability of 1.0% or less in all except two cases. The fluoroacetate group vs. the succinate group was significant at the 5% level, while succinate vs. citrate was not significant. (f) A plot of the logarithm of the mean maximum number of bacteria in each group versus the mean survival time for the mice in that group gives two straight lines with a break occurring at the mean value for succinate. This is shown in figure 1. A break gives the best curve even when the standard deviation for each mean is taken into consideration. The presence of the break, however, poses a question. Does the steeper slope hold when death results from an infection in which the initial bacteremia is overwhelming and the lesser slope when the infection is such that the initial bacteremia is transitory, as it is with the citrate and control groups, and must be followed by subsequent multiplication of the bacteria? Evidence at hand does not permit an answer.

*2. The effect of* S. typhimurium *antigen (endotoxin) and Krebs cycle inhibitors and intermediates on blood sugar concentration in mice.*—Animals given injections of endotoxins are known to develop an initial hyperglycemia followed by hypoglycemia (Boivin and Mesrobeance, 1934; Delafield, 1932; Kun and

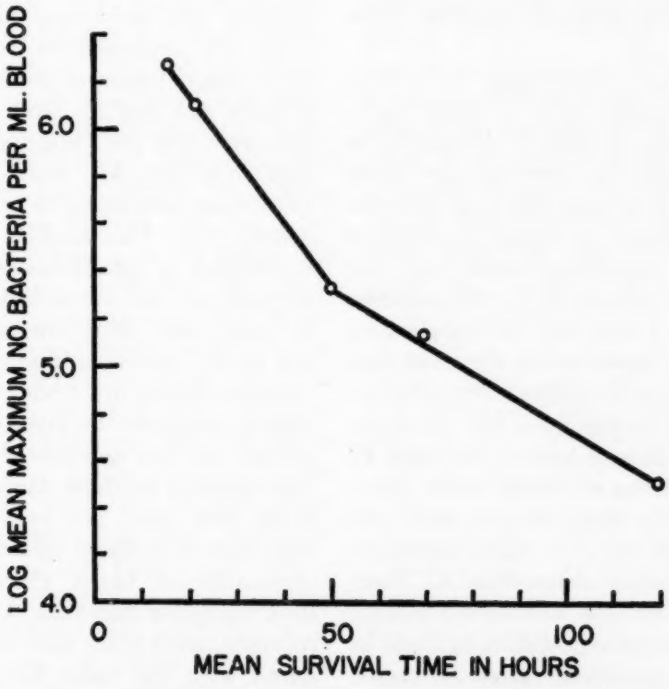


FIG. 1.—The logarithm of the mean maximum number of bacteria per ml of blood from mice of different experimental groups plotted against the mean survival time of that group. All values were taken from table 3. The points as they appear in the graph reading from left to right have the following numerical values: malonate group, log 1,796,000 vs. 14.8; fluoroacetate group, log 1,281,000 vs. 21.2; succinate group, log 210,800 vs. 50.6; citrate group, log 162,900 vs. 70.3; control group, log 31,142 vs. 110.6.

TABLE 3.—*Blood sugar concentration ± the standard deviation in mice at different intervals of time after the injection of* S. typhimurium *endotoxin. The value for normal mice is given for comparison.*

| Time after endotoxin injection | Number of mice used | Number of separate determinations | Blood sugar concentration (mg %) |
|---|---|---|---|
| Before | 30 | 14 | 163.4 ± 25.0 |
| 1 hr | 7 | 3 | 190 ± 6.0 |
| 2 hr | 6 | 3 | 142 ± 1.3 |
| 3 hr | 6 | 3 | 118 ± 19.0 |
| 4½ hr | 11 | 4 | 79 ± 8.6 |
| 6 hr | 6 | 3 | 55.6 ± 3.3 |
| 15–17 hr | 26 | 12 | 63.1 ± 7.0 |

Miller, 1945; Menten and Manning, 1924; Oddy and Evans, 1940; Zeckwer and Goodell, 1925), but the changes occurring in blood sugar of mice when both malonate and endotoxin are given remain to be determined. This becomes important since it is known (Berry, Ehlers and Mitchell, 1954) that the administration of these two substances in proper sequence leads to convulsive death in 100% of animals within a few hours.

The average blood sugar concentration in 30 normal mice is 163.4 mg%. This is shown in table 3. Presented in the same table are the average blood sugar levels of mice at different time intervals following the administration of 0.5 ml of a heat-killed brain heart infusion broth culture of *S. typhimurium*. At the end of 1 hour, the blood sugar concentration is significantly elevated, but by the 2nd hour it is below normal and it continues to decline until the 6th hour. At this time it is as low as it is after 17 hours. Injections of sterile brain heart infusion broth alone do not alter the level of blood sugar in mice; and injections of a saline suspension of thoroughly washed and heat-killed organisms, in numbers equivalent to those in the culture, produced, after 17 hours, hypoglycemia that is similar to that shown in table 3 (60.3 vs. 63.1). Therefore, the decrease in blood sugar is due to the endotoxin and not to the medium in which the organisms were suspended.

An important change in blood sugar concentration was observed following the administration of two malonate injections, each containng 20 mg in 0.5 ml of solution and the second following the first by one hour, which were started about 15 hours after the animals had been given antigen. An average value of 32.1 mg% was found in 9 mice, as shown in table 4. All of these animals were in convulsions at the time they were sacrificed and none survived the full 2 hours. This severe hypoglycemia approaches the 17.5 mg% found in 8 mice that were suffering from insulin shock at the time the blood samples were taken. These values are presented in table 4 and they are to be compared with the blood sugar concentrations obtained when arsenite, fluoroacetate, citrate, and succinate were each substituted for malonate. Arsenite injections are accompanied by the lowest blood sugar values of any of these compounds (21 mg%), and none of the mice survived the 2 hour period. Fluoroacetate is next, 47.7 mg%, followed by citrate and succinate, 55 and 57.5 mg%, respectively. This ranking is not to be considered as established since the differences are not statistically significant in most cases. Only one of the mice in the group receiving fluoroacetate succumbed during the 2-hour interval that elapsed between the injection of the inhibitor and the sacrifice of the animals. Introduction of these same compounds alone into mice not previously given endotoxin is without effect on the concentration of blood glucose. This is shown only for malonate, a result that is representative of all, and should be compared with the value for normal mice given in table 3. On the basis of this evidence, it seems reasonable to assume that the death of mice given heat-killed *S. typhimurium* followed 15 to 17 hours later by hourly injections of malonate

TABLE 4—*Blood sugar concentration ± the standard deviation in mice injected 15 to 17 hours in advance with* S. typhimurium *endotoxin and then injected with malonate, arsenite, fluoroacetate, citrate, or succinate. These values are to be compared with those following injections of malonate alone or with mice in insulin shock.*

| Conditions under which blood sugar was determined | Number of mice | Number of tests | Blood sugar concentration (mg %) | Comments |
|---|---|---|---|---|
| 15 hrs after endotoxin + malonate injections (2×20) | 9 | 5 | 32.1 ± 4.4 | None survived 2 hr after 1st malonate |
| during insulin shock | 8 | 4 | 17.5 ± 7.3 | |
| 15 hrs after endotoxin + arsenite (1×0.125 mg) | 6 | 3 | 21.0 ± 8.4 | None survived 2 hr after arsenite injection |
| 15 hrs after endotoxin + fluoroacetate (1×8 mg/kg) | 6 | 3 | 47.7 ± 5.6 | 5 survived 2 hr |
| 15 hrs after endotoxin + citrate (2×10 mg) | 7 | 3 | 55.0 ± 19.1 | 7 survived 2 hr |
| 15 hrs after endotoxin + succinate (2×20 mg) | 6 | 3 | 57.5 ± 3.1 | 6 survived 2 hr |
| 1 hr after second malonate injection (2×20) | 8 | 4 | 159 ± 14.2 | |

(Berry, Ehlers and Mitchell, 1954) is possibly related in some manner to the hypoglycemia. Certainly the convulsive death of any of these animals, regardless of the compound responsible, presents the same appearance as that observed with overdoses of insulin.

The question now arises as to why the endotoxin produces hypoglycemia. Since the animals exhibit a loss of appetite, a decrease in activity, and a definite appearance of illness, there is the obvious possibility that inanition alone is responsible. Accordingly, mice were placed in cages without food for a period of 20 to 24 hours. At the end of this time they were sacrificed and their blood sugar concentration was determined. As table 5 shows, hypoglycemia (61.4 mg%) as severe as that produced by endotoxin (table 3) was found in these animals. However, when malonate injections were made into such mice, there was no significant drop in blood sugar. One must conclude, therefore, that the decrease in blood sugar following malonate injections into mice previously inoculated with endotoxin is not dependent, per se, on an initially low blood sugar, since malonate does not lower blood sugar when it is injected into starved mice with a blood sugar equally as low. Implied in these observations is an unexplained relationship between antigen and malonate which permits the latter to lower blood sugar. As evidence that the hypoglycemia observed 17 hours after injection of endotoxin is due to depletion of carbohydrate reserves, in agreement with other workers (Kun and Miller, 1948; Koch and Olitzi, 1946; Kun, 1948a; Kun and Abood, 1949), is the fact that an injection of a quantity of adrenalin capable of raising the blood sugar level in normal mice to an average of 243.5 mg% (see table 5) is unable to alter the hypoglycemia of mice given the antigen. Adrenalin in starved mice, however, raises blood sugar to almost normal levels, proof that carbohydrate reserves remain. These conclusions regarding the

TABLE 5.—*The effect of 20 to 24 hours of starvation, of starvation followed by injections of either malonate or adrenalin, of adrenalin injections in normal mice, and of* S. typhimurium *endotoxin plus adrenalin on the blood sugar concentration ± the standard deviation in mice.*

| Conditions under which blood sugar was determined | Number of mice | Number of tests | Blood sugar concentration (mg %) |
|---|---|---|---|
| After 20–24 hrs starvation | 10 | 5 | 61.4 ± 8.7 |
| After 22 hrs starvation + 2 malonate injections | 8 | 4 | 57.3 ± 7.0 |
| After 22 hrs starvation + an injection of adrenalin | 8 | 4 | 149.3 ± 14.3 |
| 15 hrs after endotoxin + an injection of adrenalin | 8 | 4 | 56.5 ± 16.5 |
| normal mice 1 hr after an injection of adrenalin | 8 | 4 | 243.5 ± 22.6 |

carbohydrate reserves have been verified by glycogen determinations according to the method of Good, Kramer and Somogyi (1933) on the livers of normal mice, of mice 15 to 17 hours after endotoxin injections and of mice after 20 to 24 hours of starvation. Each assay was carried out on the pooled livers of two or three mice. The average values found in three or four assays with mice of each group are as follows: normal mice, 2.5% glycogen (as glucose); mice injected with endotoxin, 0.025%; and starved mice, 0.21%. According to these values, starvation reduces glycogen to about 10% of normal, while endotoxin reduces it to about 1% of normal.

Another difference between the mode of action of endotoxin and inanition in lowering blood sugar concentration in mice is made evident when the time factor is reduced from 17 to 24 hours to 4.5 hours. As shown in table 6, the blood sugar value in mice starved 4.5 hours averages 130.3 mg% and in similar mice injected with malonate it is 129.9 mg% In contrast to these values, which are lower than those for normal mice, as shown in tables 3 and 4, are the values found 4.5 hours after *S. typhimurium* antigen is injected. For such mice an average value of 79.5 mg% was found, and following malonate injections the blood sugar decreased to 48.5 mg%, a value not significantly different from that found when adrenalin was substituted for malonate. The value of 43.4 mg% suggests a depletion of carbohydrate reserves. These data prove that inanition alone cannot produce hypoglycemia within the same time period as severe as the bacterial endotoxin. This is not to say, however, that an elevated metabolic rate combined with inanition may be responsible, at least in part, for the difference.

This link between bacterial endotoxin, malonate, and hypoglycemia poses a question easily answered by a simple experiment. Can injections of sugar protect a mouse from the lethal effects of endotoxin plus malonate? The answer is a qualified yes, as shown by the results presented in table 7. Here it can be seen that 8 of 9 mice given hourly injections of malonate, starting approximately 16 hours after heat-killed *S. typhimurium* was administered, die within a period of 1½ hours. The same amount of malonate in combination with glucose fails to kill any of the animals during this same period of time, even though 4 of 9 mice die within the

TABLE 6.—*Blood sugar concentration ± the standard deviation in mice after 4½ hours of starvation, of starvation plus injections of malonate, and in mice 4½ hours after the injection of* S. typhimurium *endotoxin, of endotoxin plus malonate and endotoxin plus adrenalin injections*

| Conditions under which blood sugar was determined | Number of mice | Number of tests | Blood sugar concentration (mg %) |
|---|---|---|---|
| After 4½ hrs starvation | 13 | 6 | 130.3 ± 20.1 |
| After 4½ hrs starvation + 2 malonate injections | 7 | 3 | 129.9 ± 14.5 |
| 4½ hrs after endotoxin | 11 | 4 | 79.0 ± 8.5 |
| 4½ hrs after endotoxin + 2 malonate injections | 12 | 5 | 48.5 ± 9.8 |
| 4½ hrs after endotoxin + an injection of adrenalin | 7 | 3 | 43.5 ± 5.7 |

TABLE 7.—*The combined results of two separate experiments showing the survival of mice inoculated with about 1 billion heat-killed* S. typhimurium *followed, after 15 hrs, by hourly injections for a total of three injections with 0.5 ml of one of the following solutions: normal saline containing 20 mg malonate, 20 mg malonate and 40 mg glucose, 40 mg glucose, or saline alone. Animals given the injections but not the bacteria survived.*

| Time in hours | Mice injected with | | | |
|---|---|---|---|---|
| | Malonate | Malonate + glucose | Glucose | Saline |
| 0 | 9* | 9 | 10 | 9 |
| 1 | 5† | 9 | 10 | 9 |
| 1½ | 1 | 9 | 10 | 9 |
| 2 | 1 | 5 | 10 | 9 |
| 3 | 0 | 4 | 8 | 9 |
| 6 | | 4 | 8 | 7 |
| 24 | | 0 | 5 | 6 |

* Number at start.
† Number surviving at time interval indicated.

next 30 minutes. The last mouse injected with malonate alone dies by the 3rd hour, and the fifth mouse injected with both malonate and glucose dies during the same interval. Mice receiving glucose alone or saline alone suffer 2 and 1 deaths, respectively, within the same 3-hour period. Twenty-four hours after the start of the injections, 5 of 10 mice given glucose and 6 of 9 mice given saline survive, a difference that is not statistically significant. As further proof that endotoxin plus malonate alter the mouse's ability to metabolize glucose is the following data. A single injection of glucose into each of 4 mice 17 hours after they had been given endotoxin gave an average blood sugar concentration of 223 mg%. An equal number of mice also given endotoxin but injected with both glucose and malonate had an average blood sugar concentration of 144.5 mg%. When the injections of both glucose and malonate were repeated in such mice for a total of four injections, the average blood sugar level in three mice was 104 mg%. This indicates that these large quantitites of glucose were unable to produce a normal blood sugar concentration in mice receiving endotoxin, malonate and glucose. However, it seems very likely that the death of some of these mice occurs at a time when the blood sugar level is well above the level which results in hypoglycemic convulsions. The immediate cause of death under such conditions must be sought in other changes, possibly the ability of the animals to utilize glucose.

*3. The effect of* S. typhimurium *antigen (endotoxin) on the lactic acid content of mouse organs.*—Since previous work from this laboratory made evident an increase in citric acid in certain organs of mice previously injected with a heat-killed culture of *S. typhimurium* (Berry, Ehlers and Mitchell, 1954) and since low blood sugar does not appear to account for the death of mice injected first with endotoxin and then with malonate and glucose, the possible existence of other metabolic disturbances was sought in the assay of organs for lactic acid. Lactic acid was determined in blood, spleen, liver, kidney, heart and duodenum of normal mice and of mice 15 to 17 hours after they had been injected with either a heat-killed culture of *S. typhimurium* or a heat-killed washed suspension of the same organism. The results are summarized in table 8. There is a statistically significant decrease in the lactic acid content of liver, kidney, heart and duodenum of mice given either type of antigen (no differences were observed in their action) compared with that of normal mice. The smallest significant change was in the liver, where a value 79% of normal was obtained, while the largest change was in the duodenum, where a value 52% of normal was found. The lactic acid content of both blood and spleen was unaltered. Other workers (Boivin and Mesrobeanu, 1934; Delafield, 1932; Kun and Miller, 1948), however, using related endotoxins in larger doses, lethal for rabbits, within 2 to 3 hours, found an increase in blood lactic acid concentration. When assays were made on the blood of mice 1 hour after they had been given the same number

TABLE 8.—*Lactic acid content (in micrograms per gram wet weight), ± the standard deviation, of organs of normal mice and of mice 15 to 17 hours after the injection of heat-killed* S. typhimurium.

| Organ or tissue | Micrograms of lactic acid | | Probability |
|---|---|---|---|
| | Normal mice | Injected mice | |
| Blood | 257 ± 16.9 | 240 ± 28.7 | |
| Spleen | 434 ± 47.1 | 435 ± 36 | |
| Liver | 504 ± 35.6 | 396 ± 31 | <0.01 |
| Heart | 1680 ± 113 | 1213 ± 183 | <0.01 |
| Kidney | 681 ± 79.6 | 409 ± 67 | <0.01 |
| Duodenum | 1092 ± 73.6 | 567 ± 43 | <0.01 |

Each value in the table is the average of at least 5 separate determinations on the pooled samples of 2 to 5 mice.

of heat-killed organisms used to obtain the data of table 8, a decrease in lactic acid was found. The average of two determinations, each with 5 mice, was 179 μg per ml (174 and 183). Since typhoid is an intestinal disease, it is interesting to note that the duodenum is most profoundly affected, even though this observation may be fortuitous.

## DISCUSSION

The effect of host metabolism* on the parasite and, conversely, the effect of the parasite on host metabolism have been investigated. As the results show, each is capable of exerting a profound influence on the other. It remains problematical, however, whether the implied reciprocity of action is, in reality, a true phenomenon of the infectious process.

The overwhelming rate at which the number of *S. typhimurium* increases in the blood of mice injected with malonate or fluoroacetate bears an over-all resemblance to the increase in pneumococcal bacteremia observed in rabbits injected with cortisone, compound F or adrenocorticotrophic hormone (ACTH), as recently reported by Robinson and Smith (1953). The critical difference between the two experiments, aside from species differences, is the time factor. In the present studies the peak values appeared within about 12 hours while with rabbits and pneumococci the peaks occurred at 3 days. In order for the hormones to produce this effect in an infected rabbit, injections had to be started 5 days in advance for cortisone and compound F, or 2 days in advance for ACTH, and all were continued for 3 days postinfection. In similar experiments with mice infected with pneumococci (Kass et al, 1951), the same type of preinfection hormone treatment as well as postinfection treatment, each extending over a period of several days, is required to render the animal less resistant to the pneumococci. In contrast to the several days required in the hormone experiments are the few hours involved with mice and pneumococci when an inhibitor of the Krebs cycle is injected (Berry, Merritt and Mitchell, 1954). While the explanation for the greater susceptibility of mice with what is assumed to be an altered tricarboxylic acid cycle may be sought in hormonal effects, it must be remembered that in order for this to be true the hormones would have to act with a speed and, under selected conditions, with an extreme effectiveness (all experimental mice die within a few hours and all control mice survive completely) that has not been duplicated to the authors' knowledge by the use of hormones alone. It is possible, however, that changes in the Krebs cycle may result within a few hours in an impaired cellular defense, similar to that postulated by Thomas (1953) as the preferred explanation for the increased susceptibility of experimental animals following steroid hormone administration. This suggestion is subject to experimental verification.

The basis for the changes in blood sugar accompanying the injection of certain bacterial endotoxins is not precisely known, even though it has been demonstrated that the glycogen reserves become depleted by the time the hypoglycemia reaches minimum levels (Kun and Miller, 1948; Kun, 1948a; Kun and Abood, 1949). Evans and Zeckwer

* Evidence from the literature for alterations in the functioning of the tricarboxylic acid cycle in mice and in other animals under experimental conditions similar to those described in this paper following injections of malonate, fluoroacetate and arsenite has been cited in previous publications by the authors (Berry and Mitchell, 1953b and c). This is no proof, however, that the results we observe with infected mice under these conditions are actually to be attributed to the metabolic disturbances inferred.

(1927) attributed the hyperglycemia to central stimulation of the suprarenals via the splanchnic nerves with consequent glycogenolysis. They also found that ergotamine tartrate prevented the hyperglycemia, insulin neutralized it, and thyroidectomy decreased it. Lawrence and Buckley (1927) attributed the hyperglycemia which follows injections of diphtheria toxin to glycogenolysis and reported that the action of insulin, under these conditions, was reduced, that ergotamine lowered the rise in blood sugar level, and that both thyroid and adrenals were concerned. Soskin et al (1935) maintained that abnormal dextrose tolerance curves occurring in cases of toxemia are due to the effects of the toxin on the liver and not on the pancreas, resulting in an interference with the homeostatic mechanism of the liver. More recently, Kun (1948b), in a series of experiments with muscle extracts prepared from rats injected with meningococcal endotoxin, showed that glucose utilization was markedly inhibited and that the inhibition could be neutralized by insulin. He concluded that the activity of hexokinase was reduced by the endotoxin. Thus the observations of Soskin et al would be explained more specifically by the work of Kun.

The hypoglycemia that follows the initial rise in blood sugar level may be attributable to the combined action of several factors. Faulty glucose utilization would prevent the proper conservation of carbohydrate reserves, while the inanition and the febrile reaction, with its implied elevation in metabolic rate, would lower intake and raise output. Thus, as seen in table 6, inanition alone results in a significant reduction in blood sugar within 4 to 5 hours. Proof that this explanation alone is inadequate is the fact that malonate injections into mice previously inoculated with endotoxin result in a precipitous drop in blood sugar, while mice with an equivalent hypoglycemia produced by inanition are completely uninfluenced by malonate injections insofar as blood sugar changes are concerned. Furthermore, the accumulation of citric acid in organs of mice given the endotoxin (Berry, Ehlers and Mitchell, 1954), evidence for a block in the tricarboxylic acid cycle, and the decrease in lactic acid content of tissues of certain organs (table 8) imply disturbances in carbohydrate utilization. Much work remains in this area, however, before a clear picture of the mode of action of the endotoxin emerges. However, a possible linkage between the biochemical effect of pathogenic bacteria and the mode of action of Kreb cycle inhibitors on the infectious process begins to emerge.

## SUMMARY

1. Bacterial counts were made on the blood of groups of infected mice injected with one of the following: sodium malonate, 20 mg hourly for 8 injections (8×20 mg), sodium succinate (8×20 mg), sodium citrate (8×10 mg), and sodium monofluoroacetate, 8 mg per kg for two injections, the second following the first by 6 hours. Control mice were given hourly injections of sterile saline for 8 injections.

2. In control mice, the average number of bacteria reached a peak value at 7 hours postinfection (26,700 per ml of blood), dropped almost to zero at 12 and 24 hours and then increased exponentially to 72 hours (406,000 per ml of blood), the time at which the first casualties occurred. Mice given citrate behaved similarly except that higher average bacterial counts were obtained and deaths occurred earlier. Mice injected with the other compounds showed only a continuous increase in the severity of bacteremia during the first 12 to 24 hours postinfection. Mice given malo-

nate had an average of more than 3 million bacteria per ml of blood by 9 hours postinfection, the maximum observed, and this was maintained until all mice were dead. Their average survival time was 14.8 hours. Mice given fluoroacetate showed the second largest average number of bacteria (866,000) at 12 hours and those given succinate were next. Average survival time appears to be inversely related to the average peak number of bacteria which appears in the blood during the first 12 hours postinfection.

3. Blood sugar concentration in mice injected with about 1 billion heat-killed *Salmonella typhimurium* rises during the first hour and then drops within about 6 hours to a hypoglycemic level that remains unchanged for the next 12 hours. Comparable hypoglycemia was obtained in mice after 20 hours of starvation (about 60 mg%). Injections of malonate into such mice (or normal mice) produced no further changes in blood sugar, but when they were made into mice previously inoculated with the killed bacteria, there was an additional drop in blood sugar which terminated in hypoglycemic convulsions and death. Similar effects followed arsenite injections and, to a lesser extent, fluoroacetate, but succinate and citrate had little influence on blood sugar concentration under these conditions.

4. Nine mice injected with heat-killed bacteria followed 17 hours later with hourly injections of malonate were all dead by the 3rd hour and 8 were dead after 1½ hours, when glucose was injected along with the malonate; all of 9 mice were alive after 1½ hours; and 4 were alive after 3 hours. Glucose, therefore, has a protective action for these mice.

5. Injections of heat-killed bacteria in mice result in a statistically significant decrease after 17 hours in the lactic acid content of duodenum, kidney, heart, and liver but no change in that of blood or spleen. The maximum significant change was in the duodenum and the minimum in the liver.

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# CEREBROSPINAL NEMATODIASIS AS A PROVOKING FACTOR IN JAPANESE B ENCEPHALITIS: AN EXPERIMENTAL APPROACH

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The various studies which have led to a better understanding of the nature and cause of cerebrospinal nematodiasis of domesticated animals have been reviewed in the English language by Innes and Shoho (1952 and 1953). The name cerebrospinal nematodiasis was used by them to designate a form of acute or subacute neuroparalysis in sheep, goats, and horses which has occurred seasonally in the Far East for many years, and where it was commonly known as lumbar paralysis. The main thesis regarding causation by nematodes, belonging to the species *Setaria digitata*, stemmed from the original investigations of Japanese workers of the Korean Research Committee on Ovine Lumbar Paralysis (1939–1944). The disease is now defined pathologically as a focal form of encephalo- or myelo-malacia, with at least some degree of concomitant eosinophilic meningitis, caused by the trauma of a wandering worm (*S. digitata*), introduced into horses, sheep, and goats as infective larvae by mosquitoes.

Innes and Shoho discussed further (a) clinically inapparent, or masked, helminthic invasion of the nervous system, (b) the possibility of helminthic migration, facilitating or activating virus infection of the nervous system which otherwise might remain latent, and (c) the problem of dual virus and helminthic infections. These speculations were linked with comparable ones of Beautyman and Woolf (1951), who noted the association of an ascarid larva in the basal ganglia, along with anterior poliomyelitis of the brain stem in a child, and in which any neurological signs that might have been referred to the ascarid were entirely masked by the viral infection. Findlay and Howard (1950) have dealt with the precipitation of anterior poliomyelitis by nonspecific stimuli. Sugawa, Mochizuki, and Yamamoto (1949), during the recent epidemic years of Japanese B encephalitis (1947–1948), observed lesions of nematodal encephalomalacia in the brains of 4 out of 69 horses which had been diagnosed as cases of virus encephalitis. This might not have been a true index of the incidence of the former (or both together), for the study was not carried out with nematodiasis in mind, nor was the examination of the brains made beyond routine limits.

On the basis of the above field studies of the natural disease in animals, a clear-cut satisfactory elucidation of possible interrelationships between two separate entities (cerebrospinal nematodiasis and any of the virus encephalitides) might not be obtainable for various reasons. (1) As the malacic lesions of the former are usually localized and might be of very small dimensions, they could be easily overlooked, especially when dealing with the large brains of horses. (2) In the case of Japanese B encephalitis the lesions are often disseminated throughout brain and cord, although there is some predilection for midbrain and hindbrain (Sugawa et al, 1949); consequently, it would also be

Received for publication February 1, 1954.

Our thanks are due to Dr. J. R. M. Innes, Chief of Pathology, Medical Laboratories, Army Chemical Center, Edgewood, Maryland, for his help in the composition of this paper.

difficult to determine the initial foci of entry. (3) Tanaka et al (1945) observed malacic lesions of nematodiasis in the brains of 9 out of 100 horses. These animals had been used for serum production and had shown no signs of neurological disorder whatsoever during life. (4) In Japanese B encephalitis of both man and animals, clinically silent or masked forms of infection are also well known to occur, and this same phenomenon might occur in other neurotropic virus infections.

Without some experimental approach, perhaps no lucid answer may be possible. Our paper thus deals with studies planned with this in mind, and mainly to determine whether the balance between subclinical and fulminating Japanese B encephalitis (in experimental mice) could be tipped in favor of the latter by a prior migration of helminthic larvae into the nervous system.

## MATERIAL AND METHODS

The Nakayama strain of Japanese B encephalitis virus was used in all experiments, this strain having been isolated in Tokyo by Kasahara et al in 1935, and since maintained by intracerebral mouse passage. As adult mice are fairly resistant to this strain by extracerebral inoculation (Tsubahara et al, 1951), it was appropriate for use in the specific experiments planned, and reported here.

Virus suspensions were prepared from experimentally infected mouse brain; the tissues were homogenized and the suspension centrifuged for 15 minutes at 2000 r.p.m. to eliminate larger tissues particles. The subcutaneous method of inoculation of virus was used, as it was known to produce subclinical infections in mice.

*Toxocara canis* was chosen as the parasitic provoking factor. Larvae of this parasite have been shown to localize in the brain of experimental mice,—first by Fülleborn (1921) and Yokogawa (1923), and later by Tiner (1953a) and Sprent (1953). This same nematode is of current interest because of possible participation in production of chronic eosinophilia in children (abnormal hosts) due to visceral larva migrans (Beaver et al, 1952). Eggs were obtained from the uteri of the worms, and cultured in moist animal charcoal for approximately 3 weeks at room temperature in the warm season, before being administered. The embryonated eggs in appropriate dilutions were then given to mice through fine plastic tubes directly into the stomach.

The mice used were 7 to 11 g in body weight. They were inoculated subcutaneously with 0.3 ml of virus suspension of serial decimal dilutions, 3 to 4 days after the administration of the worm eggs. The animals were observed for the following 10 days. From mice which died, or which were killed when moribund, the brains were taken out, were cut longitudinally, and one-half of each was fixed in 10% hot formalin for histological work. The remaining halves were cut and examined for a count of the larvae, which had migrated into the central nervous system, by pressing a slice between slide and cover glass. In some cases, virus was re-identified by intracerebral inoculation into other mice. All surviving mice in the different experimental groups were killed 10 days after virus inoculation, and the brains examined as described.

## RESULTS

According to Yokogawa (1923), the clinical signs in mice of intracerebral migration of *T. canis* larva are hemoptysis, paresis, hemiplegia and circling, with death occurring 3 to 6 days after feeding the embryonated eggs (the number of the latter given not being designated). Tiner (1953a), however, reported that the mere feeding of an indeterminate number of *T. canis* eggs did not ordinarily kill mice, and the maximum length of this kind of larvae in the experimental rodent brain was less than 0.5 mm. Sprent (1953) also showed that more than 100 *T. canis* larvae could be demonstrated in the mouse brain for 1 to 6 months after the feeding of eggs, and without production of notable signs of neurological disorder. Before starting our main experiment some preliminary work was done with over 30 mice along the same lines. It was also found by us that mice showed no obvious signs of nervous disturbance after feeding less than 2000 ascaris eggs. Depression and inappetence only were noticed, but in-

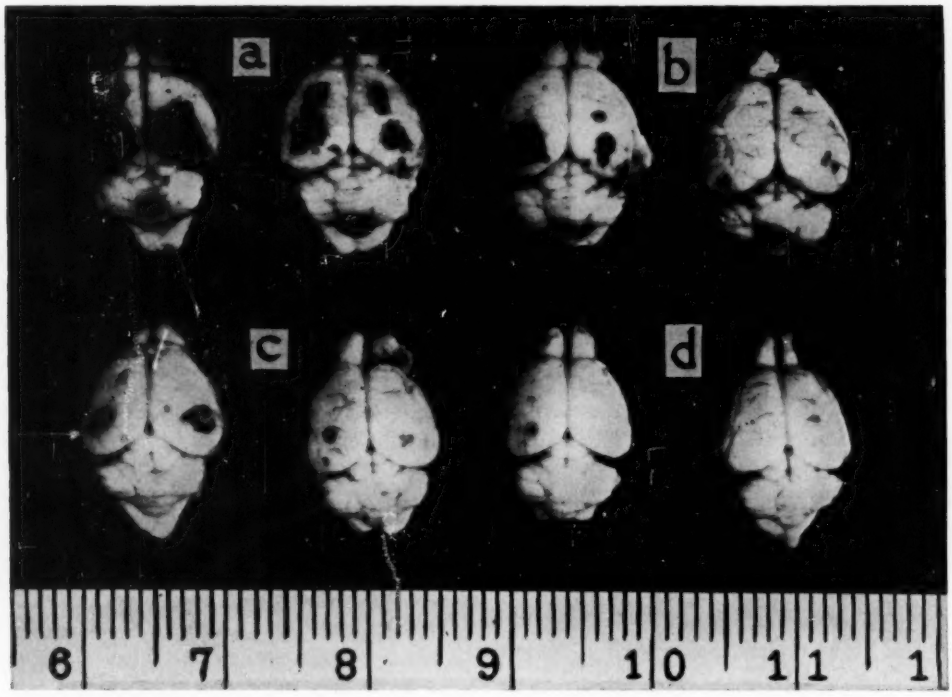


FIG. 1.—Photograph of brains of mice which had received (a) 5000, (b) 3000, (c) 2000, and (d) 500 embryonated eggs of *T. canis*. The extent of meningeal hemorrhage parallels the number of eggs given. Brains labelled *a* were from mice which died in 4 days; the remaining 6 brains, *b*, *c*, *d*, in this photo were from mice killed 6 days after feeding eggs.

tracerebral hemorrhage, if it did occur, resulted rarely in death. By feeding more than 2000 eggs, however, tachypnea, paresis, and death occurred more frequently, especially in young mice, from 1 to 6 days after feeding eggs, and apparently was attributable to severe hemorrhage in the lungs and brain (fig. 1). Cerebral migration of the larvae causing meningeal hemorrhage began in mice between 2 to 3 days after feeding the eggs. The hemorrhage became a pronounced finding at the 4 to 9 day period, and if the mice lived longer, showed as brownish spots of meningeal hemosiderosis. Macroscopically visible meningeal hemorrhage was moderate in its extent after the feeding of about 2000 eggs, was slight after only 1000 eggs, and was mostly absent when less than 500 eggs were fed to the experimental mice. Five to ten percent of the larvae hatching from the eggs generally reached the brain, and encapsulated larvae were first observed on the 11th day. This finding virtually duplicates that of Sprent (1952), who observed them in subcutaneous tissue initially on the 12th day.

*Experiment 1.*—Nineteen mice were fed about 2000 embryonated eggs; 15 of these animals and another 10 untreated mice were then inoculated subcutaneously with virus suspension 4 days later. The results obtained are shown in figures 2 and 3. A clear and significant difference in mortality was observed between the two groups of mice.

*Experiment 2.*—Twenty mice were fed about 2000 embryonated eggs, 10 with about 1000 eggs, and 10 with about 500; they were all inoculated with virus

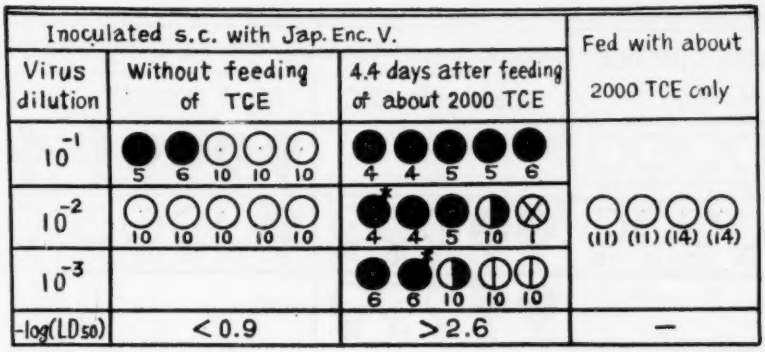


FIG. 2.—Result of experiment 1.

Black circle—died or killed in extremis, fatal nervous signs. Half black circle—killed, moderate nervous signs. Bisected circle—killed, after recovering from slight nervous signs. White circle—killed, no signs. Circle with cross—died, no encephalitis. Numerals under the circle marks indicate days died or killed after virus inoculation, and those in brackets after feeding of eggs. TCE—embryonated eggs of *T. canis*. *—recovery of virus was successfully tried by intracerebral inoculation into mice.

suspension 3 days later. Ten control mice, not fed with eggs, were inoculated subcutaneously, and another 20 untreated animals intracerebrally, with virus. The results are shown in figures 4 and 5. A tangible result was obtained even in the group of mice which had received only 500 eggs, and which later at autopsy showed only very slight meningeal hemorrhage. The detailed pathological findings will be reported later. All mice in experiment 2 which had died, or had been killed in extremis, except accidental deaths, showed typical viral encephalitic lesions. Many of the survivors, which received both virus inoculation and feeding of eggs, showed moderate lesions of encephalitis, while almost all survivors which had received only virus suspension showed very few or no lesions of encephalitis.

## DISCUSSION

The results tabulated above demon-

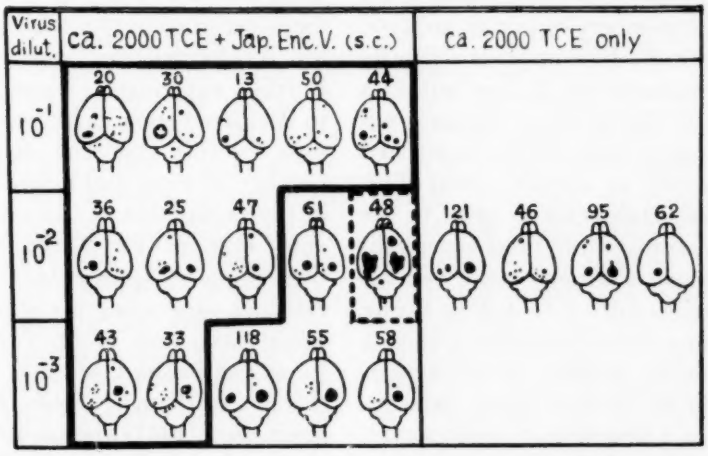


FIG. 3.—Diagram of brain, showing extent of meningeal hemorrhage and hemosiderosis in experiment 1.

Numerals indicate number of ascarid larvae found in one side of brain. Brains within thick line correspond to black circle, and in broken line to circle with cross in figure 2.

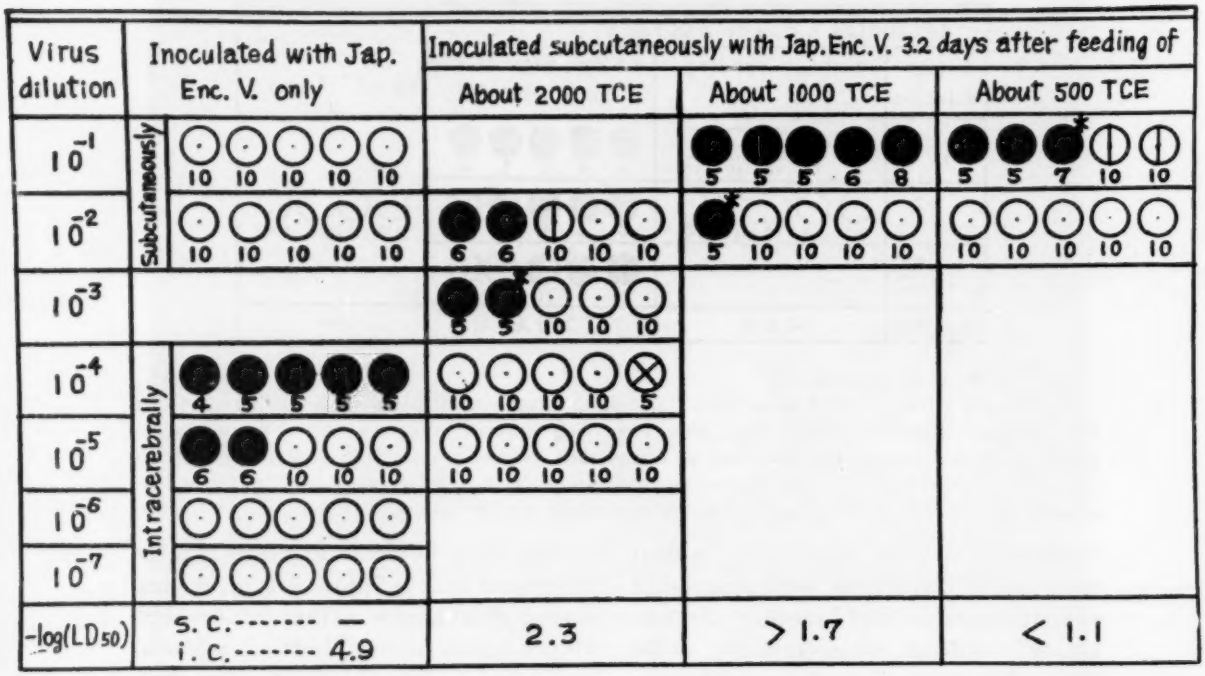


Fig. 4.—Result of experiment 2.
See explanation of figure 2.

strate (a) as indicated by the work of Tiner and of Sprent, if embryonated *T. canis* eggs are fed to abnormal hosts (mice), larvae hatch and migrate from the intestine to the nervous system, but unless large numbers of larvae migrate, they will not produce nervous signs, lesions and cause death, at least within a few days; (b) the strain of Japanese encephalitis virus used, when inoculated subcutaneously at certain serial dilutions, is not pathogenic in mice to the extent of causing acute fatal encephalitis; (c) when sufficient embryonated eggs are fed to mice 3 to 4 days before subcutaneous virus inoculation, then a very significant number of the experimental animals die from acute virus encephalitis in a few days. Consequently, it is reasonable to deduce that the migration of larvae into the brain of mice will activate or exacerbate an acute encephalitis by a later virus inoculation, which might otherwise have evoked only a masked noncerebral form. The provocation presumably then could be caused by the larvae destroying the blood-brain barrier, or by creating a malacic focus facilitating the entry of the virus for its localization and propagation. Further experiments appear necessary to decide whether there exist other reasons for this apparent pathogenic adjuvancy of virus and helminthic larvae, in the production of disease of the nervous system. From the experiments there certainly appears to be some direct evidence supporting the speculations of Beautyman and Woolf (1951) and of Innes and Shoho (1952 and 1953) along this line of thought. Sabin, Ginder, and Matumoto (1947) also mooted the probability of some intermediate arthropod-helminthic hosts for Japanese B virus being concerned in the epidemiology of Japanese B encephalitis in man.

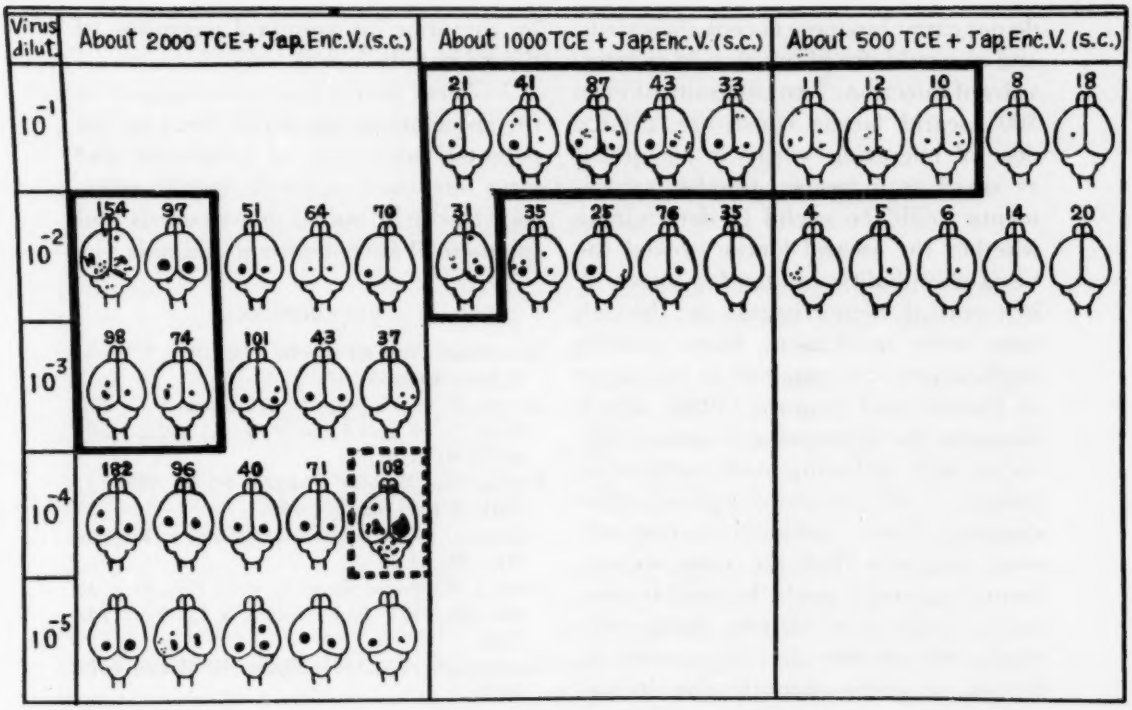


FIG. 5.—Extent of meningeal hemorrhage and hemosiderosis in experiment 2.
See explanation of figure 3.

No evidence for a concomitant virus, or other transmissible agent, was brought out in studies of ascarid larvae in rodents by Tiner (1953 a and b) and by Sprent (1952, 1953). Cerebrospinal ascariasis, which results from these larvae in the central nervous system, apparently serves to increase the probability that infected intermediate hosts (rodents) will be caught by appropriate final hosts, which are such carnivores as raccoons and badgers. By infecting mice with large numbers of larvae of the skunk ascarid (*Ascaris columnaris*), Sprent verified that they are also pathogenic in the nervous system, but only after about 17 days. This interval constitutes sufficient time for skunk ascarid larvae to grow to a length of 1 mm (Tiner, 1953). As a further contention against the presence of a virus, Tiner (correspondence, 1953) informs us that there was no fever accompanying the onset of final unconsciousness in 3 experimental rabbits infected with raccoon ascarid larvae in the brain. Histologic studies, if made, would be helpful to understand the pathogenesis of cerebrospinal ascariasis.

The relationship of *T. canis* larvae to the provocation of experimental Japanese B encephalitis in mice in these studies, must be different from that known in the case of swine lungworm to swine influenza (Shope, 1941) or from that of *Trichinella spiralis* to lymphocytic choriomeningitis (Syverton et al 1947). The pathogenic viruses causally related to these two distinct diseases are carried in a masked form by the associated worms throughout their life cycle. Yet, the potential role of *T. canis* in a parallel connection should not be underestimated, merely because apparently they

do not carry Japanese encephalitis virus, and so would not play any role as reservoirs of infection. Two thousand or even 500 ascarid larvae constitute a large dose of migrating worms in an animal as small as a mouse. Further experiments would be useful in determining whether the ascarid larvae evoked the virus encephalitis by causing shock or intracranial hemorrhages, or through some other mechanism. Some possible implications are contained in the paper of Findlay and Howard (1950), which discusses the appearance of poliomyelitis in man following such nonspecific stimuli as immunization against other diseases. Innes (personal correspondence) suggests that the same experimental approach might be used in connection with other viruses, which ordinarily only produce visceral systemic infection, yet under unpredictable circumstances become neurotropic and cause fatal acute encephalomyelitis, e.g. canine distemper and canine hepatitis, and their sometime-associated encephalitis.

## CONCLUSIONS

1. Migration of *Toxocara canis* larvae to the brain of mice was demonstrated to be effective in initiating conditions which facilitated the localization of the virus of Japanese B encephalitis in the nervous system when the latter was inoculated subcutaneously 3 to 4 days after the worm eggs had been fed by stomach tube. Alone, the strain of virus used had relatively little pathogenic effect on the brain when it was injected subcutaneously.

2. The provocation of the virus encephalitis might seem to be caused by destruction of the blood-brain barrier by the prior intracerebral migration of the nematode larvae.

3. These results give some support to the speculations made by others on the potential adjuvancy of helminthic and virus infections and with specific reference to cerebrospinal nematodiasis and Japanese B encephalitis in animals.

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# HOST FACTORS IN EXPERIMENTAL GROUP A STREPTOCOCCAL INFECTIONS
## THE ROLE OF TISSUE THROMBOPLASTIN

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Intensive study of in vitro-grown group A streptococci has resulted in the identification of a large number of products, many of which undoubtedly play some part in the pathogenesis of infection caused by this group of organisms. There is, however, no single factor or combinations of factors which can account for the great variety of tissue changes induced by these bacteria[1,2].

In a study of in vivo-grown *Bacillus anthracis*, Watson et al[3] reported that the organisms had a different metabolism than those grown in vitro; conditions found in the host resulted in the production of new factors which played a role in this infection. In addition, host-factors isolated from the tissue were thought to be produced as a result of host-parasite interaction and thereby play a significant role in the disease process.[4]

In a recent and similar study, group A streptococci were grown in an in vivo environment with the object of finding streptococcal factors of importance in the pathogenesis of this infection as well as host products which may contribute to the disease picture. A streptococcal product present in the experimental lesion was the subject of a previous report.[5] Cromartie and Watson[6,7] have reported that extracts of the experimental streptococcal lesions in rabbit skin contained a product of the host-tissue which was toxic for mice and rabbits upon intravenous injection. The rapid death and symptoms of the animals suggested that the toxic factor was tissue thromboplastin similar to that reported by Thomas.[8] Indirect evidence

Received for publication February 8, 1954.

This investigation was supported by a research grant H313 from the National Heart Institute, of the National Institutes of Health, Public Health Service.

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involving protection of animals by pretreatment with heparin, enhancement of the lethal effect by previous injection of toluidine blue, a refractive state induced by a sublethal dose, and decreased clotting time of plasma as determined by both the one and two stage techniques, indicated that the material was of a thromboplastin nature.[7] It was also demonstrated that the streptococci in the lesion extract possessed an increased virulence for mice and rabbits, and it was suggested that this might in part be accounted for by an association of the streptococci with some factor in the lesion extract such as the thromboplastic material.

This paper is concerned with the isolation and identification of this host-factor present in the experimental lesion and the evaluation of its role in the enhancement of group A streptococcal infections.

## MATERIALS AND METHODS

*Streptococcal skin lesion extract.*—The preparation and extraction of the lesion extract has been described previously.[5,7] Briefly, the technique consists of intracutaneous injection of 30 ml of an 18-hour Todd-Hewitt broth culture of group A streptococci in about 80 sites over the shaved abdomen and thorax of a 2 kg New Zealand white rabbit. Type 28 was used in the experiments reported here. The moribund animals were killed 18 hours after injection of the streptococci, the lesion ground in a meat grinder and extracted overnight at 2 C by stirring with 1 to 2 ml of saline per gram of tissue. It was then filtered through gauze, centrifuged for 1 hour in the cold at 4000 r.p.m., and filtered through Selas 10, 01, and 02 filters. The thromboplastin or "toxin" could be precipitated in the euglobulin fraction by dialysis of the extract against distilled water. To obtain the tissue thromboplastin or "toxin" in a more purified form, the extract was centrifuged for 1 hour in the Spinco 40 head at 40,000 r.p.m. (145,000 G), and the sediment resuspended in borate buffer pH 8.3 and $L/2$ of 0.15. To obtain a more purified product, the centrifugation procedure was repeated twice.

*Normal skin extract.*—Sterile broth was injected intracutaneously in place of the streptococcal culture. The skin was then excised and treated exactly as the streptococcal lesion extract, with the exception that after grinding and the addition of saline it was then subjected to sonic disintegration for 30 minutes in a Raytheon 9 KC. oscillator.

*One-stage clotting time determination.*—The method described by Quick[9] was followed. Rabbit plasma was obtained by adding 1.0 ml of 0.1 *M* sodium oxalate to 9.0 ml of blood. One-tenth ml of plasma with 0.1 ml of the extract were mixed in an 8×75 mm tube and placed in a 30 C water bath. The reaction was timed from the addition of 0.1 ml of a 0.2 *M* solution of calcium chloride. All reagents were allowed to equilibrate at bath temperature before use. The tubes were tipped gently at 10-second intervals and the end point determined by the observation of a solid clot. A clotting unit is defined as the smallest amount required to give a clotting time of at least 20 seconds less than the control.

*Titration of lethal activity of skin extracts.*—The MLD of an extract is the minimum volume which will kill at least 80% of the mice upon intravenous injection. The animals usually died within 30 seconds to 2 minutes. A comparison of lethal activity was made on the basis of the total number of MLD's present in each extract. For this reason comparable skin areas were always removed from rabbits of the same age and weight.

## RESULTS

The most active toxic material was isolated by high speed differential centrifugation similar to the technique used by Chargaff[10] to isolate thromboplastin from beef lung. This technique is described under methods. An electrophoretic analysis of the purified material was made in veronal buffer pH 8.6

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9. Quick, A. J. 1935, The prothrombin in hemophilia and in obstructive jaundice. J. Biol. Chem. **109**: Proceedings, lxxiii–lxxiv.
10. Chargaff, E., Moore, D. H. and Bendich, A. 1942, Ultracentrifugal isolation from lung tissue of a macromolecular protein component with thromboplastic properties. J. Biol. Chem. **145**: 593–603.

and $L/2$ of 0.1. Only one boundary was observed and this had a mobility of 8.1 $\times 10^{-5}$ cm²/v/sec. To identify further and to determine the purity of this product, an analysis of the lipid phosphorous and total nitrogen was carried out. The lipid phosphorus was extracted by the method of Bloor[11] and quantitated by the technique described by Boyd.[12] Total nitrogen was determined by the Kjeldahl method. The average ratio of nitrogen to lipid phosphorus was 12.4 for the material obtained from the streptococcal lesion and 14.5 for that isolated from normal control skin. These values are close to those reported by Chargaff[10] for tissue thromboplastin.

These results identify the toxic material occurring in the streptococcal lesion as thromboplastin. As indicated above, this material is present in normal as well as the infected tissue. Several experiments were performed to demonstrate if there were any quantitative or physical difference between the materials obtained from the two sources. In preparing these extracts 4 rabbits of the same age and weight (2 kg) were used. Two received 30 ml each of an 18-hour Todd-Hewitt broth culture of type 28 group A streptococci. The other two received an equal volume of the sterile broth. After 16 hours the rabbits were killed and the skin removed and extracted as usual. The extracts were divided into 2 lots and centrifuged in the Spinco 30 head for one-half hour. One lot was spun at 12,000 r.p.m. (17,000 G) and the other at 25,000 r.p.m. (73,000 G). The supernatant fluid in each case was injected intravenously into mice. Table one summarizes the results of a typical experiment. As this experiment indicates, there is initially a slightly greater activity in the normal tissue extract, as shown by the fact that after centrifuging at 12,000 r.p.m. there are a total of 430 MLD's in the normal extract supernatant compared with 328 in the lesion extract. However, after centrifuging at 25,000 r.p.m. it is observed that about twice as much activity remains in the streptococcal lesion extract supernatant. Evidently any injury of the host cells results in the release of this material. The destruction of cells by the streptococci and release of this factor is simulated by the grinding and stirring of normal tissue. However, the active materials obtained from the two sources, which are similar electrophoretically and in chemical constitution as well as biological activity, differ with respect to sedimentation rates. The active fraction seems to consist of large molecules whose sedimentation rates vary in a continuous scale from those sedimenting at 20,000 G to those sedimenting only at 140,000 G. The material obtained from the normal skin has more of its activity sedimented at 20,000 G and that obtained from the streptococcal lesion contains more of the molecules of lower molecular weight.

This host material is present in an active form in the lesion and is not an artifact resulting from the mechanical destruction of the tissue. This is indicated by the fact that the active material could be found in the edema fluid which was allowed to drain off the cut skin with no pressure applied. In this experi-

TABLE 1.—*Comparative sedimentation of thromboplastin in lesion extract and normal skin extract.*

| Preparation | Total number of mouse MLD's in extract | | Total number of clotting units in supernatant at 25,000 r.p.m. |
|---|---|---|---|
| | Supernatant 12,000 r.p.m. | Supernatant 25,000 r.p.m. | |
| Lesion extract | 328 | 272 | 14,400 |
| Normal skin extract | 430 | 143 | 8,400 |

11. Bloor, W. R. 1943, Biochemistry of Fatty Acids, New York, Reinhold Publishing Corp.
12. Boyd, E. M. 1936, The extraction of blood lipids. J. Biol. Chem. **114**: 223–234.

ment 30 ml of edema fluid was collected from 3 rabbits injected in the usual manner with type 28 streptococci. It was centrifuged at 30,000 r.p.m. for ½ hour and the euglobulin fraction precipitated upon dialysis of the supernatant against distilled water was resuspended in 5.0 ml of buffered saline. Intravenous injection of 0.2 ml of this suspension killed 6 out of 6 mice. Previous intravenous administration of 0.1 mg of heparin resulted in none out of 6 killed.

To substantiate further the observation that this material is present in an active form in the streptococcal lesion, the following experiment was performed. Two rabbits of the same age and weight were used. One was injected intradermally with 30 ml of an 18-hour broth culture of type 28 streptococci. The second animal was injected with sterile broth. After 17 hours the animals were killed and the skin removed and cut into ½ inch squares. The cut skin was allowed to infuse overnight with occasional stirring in 60 ml of saline. These extracts were then filtered through gauze, centrifuged at 4000 r.p.m. for ½ hour and filtered through a Selas bacterial filter. Neither the lesion extract nor the normal skin extract was lethal for mice upon intravenous injection. By the one-stage in vitro clotting technique a considerable difference in activity could be demonstrated. Table 2 shows these results. These extracts were then concentrated by lyophilization, resuspended in 5.0 ml of distilled water, and dialyzed against 1.0% saline. Upon intravenous injection into mice the usual lethal effect of the extract could be observed with 0.5 ml of the streptococcal extract but not with 1.0 ml of the normal tissue extract. These experiments indicate that the lethal factor, or thromboplastin, in the lesion extract is released in response to injury by the streptococci.

TABLE 2.—*Relative clotting activity and lethal effect of thromboplastin present in infusion extracts of the streptococcal lesion and normal skin.*

| Preparation | Total clotting units in extract | MLD in mice* |
|---|---|---|
| Streptococcal lesion extract | 4000 | 12 |
| Normal skin extract | 700 | 0 |

* Total number of MLD's present in the whole extract after concentration.

We next wished to determine if this host factor present in the streptococcal lesion contributed to the infectious process. As noted above, infection of rabbits occurred much more readily and endocarditis was more frequently observed with organisms obtained from the skin lesions than with in vitro-grown cultures of group A streptococci. It is conceivable that the thromboplastin, coexisting with the streptococci, produces a protective fibrin network around the organisms, allowing them to establish an infection. This could, in part, account for the apparent increased virulence of streptococci obtained from the skin lesions.

The following experiment was performed to test this hypothesis. White mice were injected intraperitoneally with 0.1 ml of a 24-hour broth culture of group A streptococci which had been mixed with 0.1 ml of the thromboplastin isolated from the lesion extract. This volume contained 4 mouse MLD's. A control group received the same culture of organisms with saline. A second control group received thromboplastin with saline. A comparison of the accumulative percent deaths seen in figure 1 demonstrates that the virulence of the organisms was definitely increased when they were associated with the thromboplastin. These results are a summary of 5 experiments with a total of 45 mice in each group. To confirm the fact that the mice were dying from a streptococcal

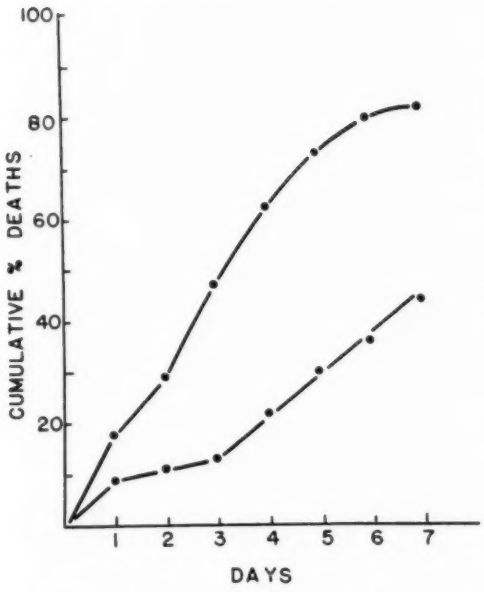


FIG. 1.—Comparative virulence of group A streptococci when suspended in saline and thromboplastin prior to intraperitoneal injection in mice. Upper curve, thromboplastin; lower curve, saline.

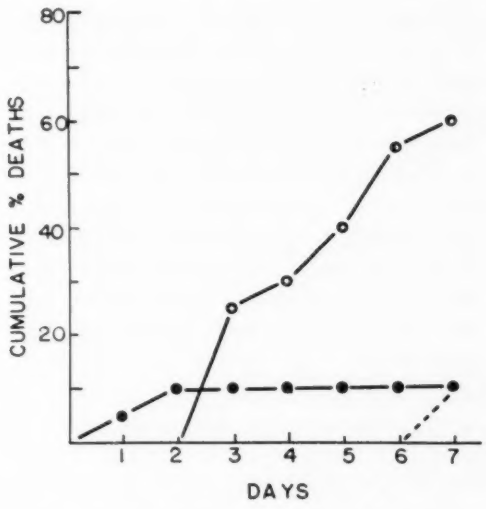


FIG. 2.—Neutralization of the virulence enhancing property of thromboplastin by heparin as manifested by the intraperitoneal injection with group A streptococci in mice.—○—thromboplastin; —●—thromboplastin and heparin; --- saline.

infection, representative animals dying in each group were examined for the presence of streptococci. Impression smears of the spleen, as well as blood agar plates streaked with spleen and heart blood, revealed a large number of streptococci present in the tissue.

An additional control was run to determine if the enhancement of the virulence was due to the clotting activity of the thromboplastin or merely to the presence of protein vehicle. This consisted of a third group of mice which were injected with the thromboplastin and organisms with an amount of heparin (0.2 mg) which was found to neutralize the thromboplastin activity. Figure 2 indicates that the effectiveness of the thromboplastin in enhancing the virulence was almost completely neutralized by the heparin.

Menkin[13,14] has worked extensively with inflammatory exudates produced with sterile irritants such as turpentine. One of the factors which he isolated, termed necrosin, has the property of producing inflammation and necrosis as well as lymphatic blockade. Some of the properties of necrosin resemble those of the toxic material in the lesion extract which we have determined to be tissue thromboplastin. The thromboplastin isolated from a skin lesion and bovine hemoglobin used as a control were diluted to contain 6.3 mg of nitrogen per ml, and 0.5 ml of each was injected intradermally into the abdominal skin of a rabbit. The thromboplastin gave a reaction similar to that described by Menkin[15] as typically produced by necrosin. Edema, erythema and in-

13. Menkin, V. 1943, Chemical basis of injury in inflammation. Arch. Path. **36:** 269–288.
14. Menkin, V. 1950, Newer Concepts of Inflammation, Springfield, Ill., Charles C Thomas.
15. Menkin, V. 1950, Presence of a necrosin-like fraction in the extracts of injured tissue. Proc. Soc. Exper. Biol. & Med. **75:** 350–352.

duration were observed. Tissue sections revealed infiltration of polymorphonuclear leukocytes.

According to Menkin[16] the best criterion for necrosin is the ability to produce a lymphatic blockade. The thromboplastin was isolated from two streptococcal lesion extracts and one normal skin extract by high speed centrifugation, and resuspended in the borate buffer. The total nitrogen content of these suspensions varied from 1.5 to 2.1 mg per ml; the protein concentration was thus calculated to be approximately 1.5%. Sterility was tested by streaking a blood agar plate. One ml was injected intradermally into the right foreleg of a rabbit, and after 3 hours edema and erythema were evident signs of inflammation. A small amount of the fluid was aspirated and streaked on blood agar plates; no growth occurred after either aerobic or anaerobic incubation. A similar volume of normal rabbit serum was injected into the other foreleg and no evidence of inflammation resulted.

After 6 hours in the first experiment and 24 hours in the second and third experiments, 0.5 ml of 1.0% trypan blue was injected into the area of inflammation. Within 2 hours the trypan blue had spread diffusely in the control leg. Very little diffusion of the dye was observed in the inflamed leg. This comparison is illustrated in A and B of figure 3. The regional lymph nodes were removed to determine the difference in the amount of dye which had drained from the sites of injection. It was difficult, however, to assess with any accuracy the amount of dye which had reached the nodes.

These results indicate that the necrosin described by Menkin may be a large lipoprotein molecule with thromboplastic activity or at least associated with such a molecule. Menkin has published no physical or chemical studies of necrosin other than some determinations of nitrogen and phosphorus content of the crude euglobulin fraction of the exudate. Thus, no comparison can be made on this basis, although the following points can be considered: both are precipitated at 33 to 50% saturation with ammonium sulfate and by

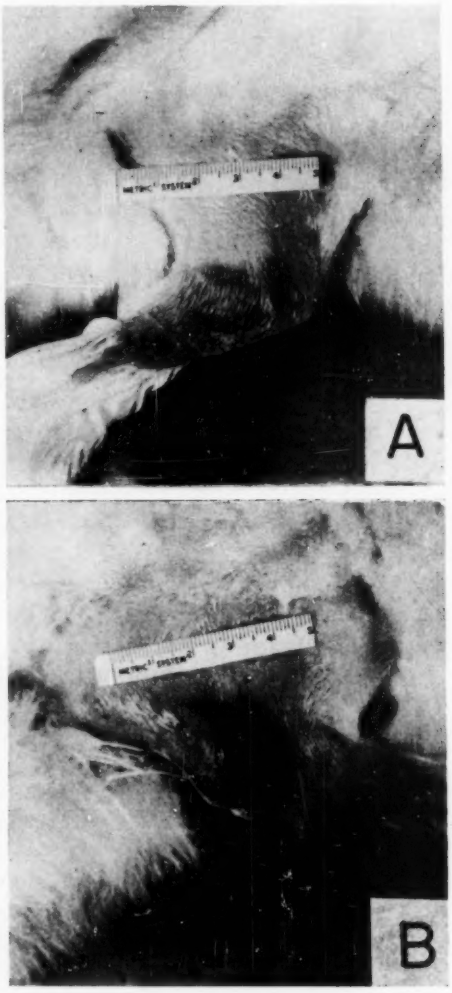


FIG. 3.—A. Left foreleg of rabbit 1 hour after intradermal injection of 0.5 ml of 1.0% trypan blue. The dye was injected 24 hours after injection of normal rabbit serum. B. Right foreleg of the same animal 1 hour after intradermal injection of 0.5 ml of 1.0% trypan blue. The dye was injected 24 hours after injection of thromboplastin prepared from a streptococcal lesion extract.

16. Menkin, V. Personal communication.

dialysis against distilled water, and both can produce a necrotic process in the skin and a lymphatic blockade.

## DISCUSSION

The toxic factor in the streptococcal lesion extract is a product of the host-tissue since a material with similar properties is readily obtained from a normal skin extract, as well as from turpentine lesion extracts. These properties are similar to those of tissue thromboplastin as described by Chargaff[10] and Thomas.[8] Isolation of the active factor was carried out by high speed differential centrifugation, and it was found to have an electrophoretic mobility and chemical composition similar to that reported for purified tissue thromboplastin.[10]

The release of a material with similar properties from damaged tissue has been reported previously by Stoner and Green[17] and Menkin[15]. Two experiments demonstrate that the thromboplastin is released in response to group A streptococcal infection and is present in an active form in the lesion rather than occurring as an artifact produced in the extraction process. These involve the demonstration of the thromboplastin in the edema fluid of the lesion before any mechanical damage had occurred, and a comparison of the amount of thormboplastin obtained from a streptococcal lesion with that obtained from normal skin, both extracted with a minimum of mechanical damage.

The virulence of streptococci for mice was enhanced by injecting them with the thromboplastic fraction from either normal or lesion extracts. This supports the hypothesis that the increased virulence and incidence of endocarditis observed with organisms from the lesion extract is in part the result of the association of the streptococci with some material from the lesion. The virulence-increasing capacity of the thromboplastin could be inhibited by neutralization of the clot-promoting activity with heparin.

These results are interesting in view of the work of Lampert and Richley,[18] who demonstrated that the effect of mucin in aiding the infection of a Gram-negative organism such as *Salmonella typhosa* was due to the anticoagulant activity of its heparin-like component as well as some anticomplementary effect. In our experiments the heparin did not enhance the virulence of the Gram-positive streptococci, but actually protected the animals against the additive effect of thromboplastin.

Our findings are in agreement with the report of Evans, Miles and Niven,[19] who found that localization of the invading organism enhances the infection and dispersion aids the host. They produced an ischemia at the site of an infection by injection of adrenalin to produce vasoconstriction. This prevented a dispersion of the organisms, which is evidently a better defense mechanism against some infections than localization, since, by this technique, they noted a considerable enhancement of the infectivity of certain Gram-positive rods.

The relationship of thromboplastin to necrosin obtained by Menkin[13,14] from inflammatory exudates is evident. The fact that they have similar solubility properties, the same lethal effect in

17. Stoner, H. B. and Green, H. N. 1951, Bodily reactions to trauma. Further studies on the effect of ischemia on the clotting factor of muscle. Brit. J. Exper. Path. **32**: 183–194.

18. Lambert, H. P. and Richley, J. 1952, The action of mucin in promoting infections: the anticomplementary effect of mucin extracts and certain other substances. Brit. J. Exper. Path. **33**: 327–339.

19. Evans, D. G., Miles, A. A. and Niven, J. S. F. 1948, The enhancement of bacterial infection by adrenalin. Brit. J. Exper. Path. **29**: 20–39.

mice, and are comparable in the production of a lymphatic blockade indicates that necrosin is thromboplastin.

SUMMARY

A host factor which contributes to the disease process has been isolated from experimental group A streptococcal lesions. This factor, released by the host-tissue in response to the streptococcal injury, was identified as tissue thromboplastin. It was shown to be present in an active form in the lesion and not produced as a result of mechanical damage to the tissue. The virulence of streptococci for mice was increased by injecting this host-factor (thromboplastin) with the organisms. The thromboplastin isolated from the streptococcal lesion has several properties in common with necrosin and it is suggested that they are identical.

# THE PRODUCTION OF FUSOSPIROCHETAL INFECTIONS IN GUINEA PIGS WITH RECOMBINED PURE CULTURES

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The production of fusospirochetal abscesses in guinea pigs with recombined mixtures of pure cultures isolated from the exudates was reported in the early literature by Kritchewski and Seguin (1920),[1] Smith (1930),[2] and Proske and Sayers (1934).[3] In these studies individual cultures were inadequately identified and represented only a portion of the range of species recoverable from fusospirochetal infections in the guinea pig. On the other hand, Rosebury and others[4–6] more recently reported failure in repeated attempts to produce typical transmissible subcutaneous fusospirochetal infections in guinea pigs with mixtures of pure cultures derived either from fusospirochetal infections in guinea pigs or from gingival and periodontal lesions in man which serve as source for experimental infections in guinea pigs. These workers concluded that "the problem is more complex than would appear from the published record."[4] They suggested the following possibilities to account for these failures: (a) the recombined cultures were inadequately concentrated to produce infections; (b) certain strains suffered a loss of virulence as a result of artificial cultivation or prolonged handling; (c) mutual antagonism existed between certain of the cultured strains; (d) a virus or comparable agent was responsible for or contributed to the pathogenicity; (e) all the essential organisms were not cultivated; or (f) loss of viability of certain strains occurred during the process of recombination. Of these possibilities, all but the last two were convincingly disposed of by the experimental procedures. The possibility that some essential organism or organisms could not be cultivated could not be satisfactorily eliminated. However, evidence was presented indicating that (a) all the essential organisms could be cultivated *as a mixture* on artificial mediums through at least ten passages, and (b) all the morphologic forms appearing in exudate were recovered in pure cultures.

The results of these attempts to recombine organisms isolated from guinea pig fusospirochetal exudate did not provide evidence that all cultures grew in the recombined mixtures. The technique of recombination consisted of pooling one drop of centrifuged sediment of each strain to provide several different combinations of organisms. These pools were then inoculated as unstreaked drops on blood agar plates or into the well of spirochete cup plates.[7] This pro-

Received for publication February 8, 1954.

Supported by a grant from the Associate Committee on Dental Research of the National Research Council (Canada).

1. Kritchewski, B. and Seguin, P. 1920, Rev. de Stomatol. **22**: 41, 613–647.
2. Smith, D. T. 1930, J. Infect. Dis. **46**: 303–310.
3. Proske, H. O. and Sayers, R. R. 1934, Pub. Health Rep. **49**: 839–858; 1212–1217.
4. Rosebury, T., Clark, A. R., Engel, S. G. and Tergis, S. 1950, J. Infect. Dis. **87**: 217–225.
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7. Rosebury, T., Macdonald, J. B., Ellison, S. A. and Engel, S. G. 1951, Oral Surg., Oral Med. & Oral Path. **41**: 68–85.

cedure did not disclose whether the strains in each recombination mixture all grew. In a previous experiment[5] resuspended sediments from centrifuged cultures were combined quantitatively (according to the proportions of the various forms in exudate) and inoculated directly into guinea pigs without further cultivation. Emphasis was placed on maintenance of virulence, and to this end the earliest available pure culture of each strain was preserved in carbon dioxide ice until used in all these recombination experiments. This procedure apparently was not necessary because mixed material was later shown to suffer no loss in virulence on repeated subculture.

The present report deals with successful attempts to reproduce fusospirochetal infection in guinea pigs with mixtures of pure cultures. It differs from the studies reported by Rosebury and his coworkers[4–6] in that particular attention was given to the problem of assuring viability of strains at the time of recombination.

## I. INITIATION OF GUINEA PIG INFECTION AND ISOLATION OF STRAINS

### *Procedure*

The source of material for guinea pig inoculations was the mouth of a 68-year old patient (HG) with a clinical diagnosis of hypertrophic gingivitis. Material from gingival crevices and periodontal pockets was collected with sterile scalers and suspended in 1 ml of Hartley broth.[8] Darkfield examination of this material disclosed a typical mixed oral flora as described elsewhere,[9] including a variety of actively motile spirochetes, several forms of motile rods and spirilla, nonmotile rods and filaments, some of which showed tapered ends, and numerous cocci. This suspension was inoculated subcutaneously in a dosage of 0.35 ml into the groin of 2 young adult guinea pigs. The tissues were deliberately traumatized with the needle at the time of inoculation. Pus aspirated on the 8th day from a 1-inch localized abscess of one of these animals was passed without trauma to 2 more guinea pigs in a dosage of 0.1 ml. Repetition of this procedure through 4 passages, accompanied each time by a darkfield examination of the pus and aerobic cultivation on sheep blood agar, resulted in the development of an exudate from which only 2 aerobic organisms could be cultivated—a nonhemolytic streptococcus and a diphtheroid-like form. All other cultures were isolated and grown under anaerobic conditions in hydrogen Brewer jars containing 5% carbon dioxide.

The mediums used were similar to those used in earlier studies[4–6] and included the following: (1) Ten percent sheep blood agar prepared with Bacto beef extract (no. B131), Bacto heart infusion (no. 338) or Proske-Sayers veal infusion base[3] and 1.5% agar. (2) Ascitic fluid-kidney extract mediums prepared with either Bacto heart infusion base (HIAFKE medium) or with Proske-Sayers base (PSAFKE medium). These have been previously described.[10] They contained 30% ascitic fluid, 0.1% neutralized cysteine hydrochloride and 5% aqueous extract of fresh guinea pig kidney. They were prepared either as broth or with 1.5% agar. (3) Proske-Sayers agar enriched with 30% ascitic fluid and pieces of guinea pig kidney (PSAFK agar) for use in Noguchi tubes. This medium, described previously,[7] was used for cultivation of spirochetes. (4) Difco thioglycollate broth (no. B236). (5) Hartley broth.[8]

Strains were isolated from the 1st, 4th, 5th, 6th and 7th guinea pig passages. Exudates were either streaked directly on solid medium or first cultured through one or more passages as unstreaked drops on solid medium. Spirochetes were isolated by inoculation of exudates into the well of spirochete cup plates or by inoculation of blood agar plates (prepared with Proske-Sayers base) as unstreaked drops. In the first instance spirochete-laden agar from the haze surrounding the well was aspirated by capillary pipette and transferred to the depths of Noguchi tubes containing PSAFK agar.[7] In the second instance where blood plates were inoculated as unstreaked drops, a peripheral fringe of spirochetes free from bacteria appeared in 5 to 7 days. This fringe varied in width from 0.5 to 1.0 cm. Some of this fringe of

8. Stitt, E. R., Clough, P. W. and Clough, M. C. 1938, Practical Bacteriology, Haematology and Animal Parasitology, Philadelphia, P. Blakiston Son and Co.
9. Rosebury, T., Macdonald, J. B. and Clark, A. R. 1950, J. Dent. Res. **29**: 718–731.
10. Macdonald, J. B. 1953, Motile Non-sporulating Anaerobic Rods of the Oral Cavity, University of Toronto Press.

spirochete growth was emulsified in a tissue grinder with a small amount of sterile broth. The emulsified growth was then transferred by capillary pipette to Noguchi tubes of PSAFK agar.

All cultures (except spirochete cultures in Noguchi tubes) were incubated anaerobically at 37 C for 5 days to 2 weeks. Noguchi tubes were incubated aerobically. All pure cultures were stored in carbon dioxide ice in sealed 1-ml glass ampules.

Twenty-nine pure cultures isolated from HG exudates (excluding cultures of spirochetes) were tested to determine replication and for provisional identification of the strains. Tests included cultural and morphological examination and biochemical tests. Each culture was grown on blood agar and in Difco thioglycollate broth (B236) and PSAFKE broth. Cultures in sugar-free thioglycollate (BBL) broth containing 1% of dextrose, sucrose or mannite, pH 7.2, were tested for fermentation with a glass electrode potentiometer. Production of hydrogen sulfide was determined in a medium prepared from equal parts of PSAFKE agar and Difco peptone iron agar. Formation of indol was tested in Difco thioglycollate broth by shaking 1 ml of xylol with the culture, heating to 55 C, and adding 7 or 8 drops of Ehrlich's reagent.[11] The method of Topley and Wilson[12] was used to test nitrate reduction in cultures in BBL broth containing 0.1% potassium nitrate. Addition of 0.0001% sodium nitrite to BBL broth gave a distinctly positive test. Acid production in litmus milk was recorded, and the solubility in 10% sodium hydroxide of the clot, if any, was tested.[12]

Negative controls of all tests were performed with sterile broth. Inoculation in all cases was by capillary pipette, using 5 to 10 drops, depending on the turbidity of the seed culture. Peptone iron agar tubes were inoculated by stabbing the solidified medium. Cultures were incubated anaerobically for periods ranging from 3 to 7 days, depending on the rate of growth of each strain. All tests were repeated and confirmed at least once.

## *Results*

By eliminating duplications among the strains isolated, the number of distinctive strains appeared to be 17. This number included one "strain" of spirochetes which can be considered pure only in the sense that it was separated from other bacterial forms. The methods of isolation of spirochetes, as noted elsewhere,[7] made it seem likely that all of several cultures isolated would be comparable. No "double haze" as described by Rosebury and his coworkers[7] was observed. Darkfield appearance was uniform from one culture to the next. Thin, tightly wound spirochetes of the *T. microdentium* variety with approximately 10 curves predominated. Both longer and shorter forms were also found. Some shorter forms with 5 or 6 curves were slightly thicker and irregularly wound. Consequently each culture of spirochetes isolated was considered to include all the cultivable forms.

The characteristics of the distinctive strains (exclusive of spirochetes) are listed in table 1. As in earlier studies,[4–6] they fall into 5 morphological groups: (1) streptococci, (2) gram-negative nonmotile rods, (3) gram-negative motile rods, (4) fusiform rods, and (5) gram-positive nonmotile rods.

*Streptococci.*—Seven strains of streptococci were isolated. One only (JSI) was aerobic and was identified as *S. mitis*. It produced slight greening on blood agar and failed to produce mucoid colonies on 5% sucrose agar.[13] The other 6 strains were all anaerobes. Three strains (JR4, JR5 and JS9) appeared similar in all respects except for fermentation of mannite (positive for JR5, negative for JR4, JS9) and production of acid in litmus milk (negative for JS9, positive for JR4, JR5). The remaining three strains were unique in at least one respect, i.e., colonial form of K98 and K112, and biochemical inactivity of K100.

*Gram-negative nonmotile rods.*—Three

11. Frobisher, M., 1940, Fundamentals of Bacteriology, Philadelphia, W. B. Saunders and Company.
12. Topley, W. W. C. and Wilson, G. S. 1946, Principles of Bacteriology and Immunity. Baltimore, The Williams and Wilkins Company.
13. Niven, C. F., Smiley, K. L. and Sherman, J. M. 1943, J. Bact. **45**: 479–484.

TABLE 1.—*Characteristics of pure cultures isolated from HG guinea pig exudate.*

| Strain | Colony on blood agar | Growth in broth | | Cell morphology | Gram stain | Fermentation | | | Hydrogen sulphide | Indol | Litmus milk | Nitrate |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | PSAFKE | B236* | | | Dextrose | Sucrose | Mannite | | | | |
| JS1† | Pinpoint, smooth, grey, convex | ++ | +++ | Cocci-short chains | + | 4.3 | 4.4 | 0 | 0 | 0 | Ac. Cl. | 0 |
| JR4 | Pinpoint, smooth, grey, convex, opaque | + | +++ | Cocci-short chains | + | 4.9 | 0 | 0 | 0 | + | Ac. | + |
| JR5 | ½ mm., smooth, grey, convex, opaque | + | +++ | Cocci-short chains | + | 5.6 | 0 | 5.5 | 0 | + | Ac. | + |
| K98 | ½ mm., smooth, yellow, convex, opaque | ++ | ++ | Cocci-short chains | + | 6.4 | 6.6 | 0 | 0 | 0 | 0 | 0 |
| K100 | ½ mm., smooth, grey, convex | +++ | ++ | Cocci-long chains | + | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| K112 | ½ mm., smooth, pink, marginal depression | + | ++ | Cocci-short chains | + | 5.7 | 6.7 | 0 | 0 | 0 | 0 | 0 |
| JS9 | ½ mm., smooth, grey-white | + | +++ | Cocci-short chains | + | 5.4 | 0 | 0 | 0 | + | 0 | + |
| JR6 | ½ mm., smooth, grey, translucent | + | + | Ovoid rods | 0 | — | — | — | 0 | 0 | Ac. | — |
| K92 | 2 mm., creamy, heaped greening | + | + | Short to long bent rods | 0 | 6.2 | 0 | 0 | 0 | + | 0 | 0 |
| K110 | ½ mm., smooth, olive-green to black | 0 | 0 | Irregular rods 1 to 6μ | 0 | | | | | | | |
| JV5 | ½ mm., flat, mottled, grey, greening | + | + | Slender rods, 2–6μ, darting motility | 0 | 0 | 0 | 0 | + | 0 | 0 | 0 |
| K80 | 1 mm., flat, mottled, iridescent | ++ | ++ | Straight thick rods, 6–8μ, sluggish motility | 0 | 6.5 | 0 | 0 | — | + | 0 | + |
| JF5 | ½ mm., low convex, grey mottled | + | ++ | Tapered rods 6 to 8μ | 0 | 6.1 | 6.1 | 0 | 0 | + | Ac. | 0 |
| JR3† | ½ mm., smooth, creamy, opaque | + | ++ | Irregular rods 4 to 30μ | + | 4.6 | 4.6 | 0 | 0 | 0 | Ac. Cl. | 0 |
| JB3A | ½ mm., smooth, grey, translucent | + | + | Ovoid rods | + | 0 | 0 | 0 | 0 | 0 | 0 | + |
| JB3B | ½ mm., smooth, creamy, opaque | +++ | +++ | Rods, 2 to 4μ | + | 4.9 | 0 | 0 | 0 | + | Ac. | + |

For growth in broth, 0 = no growth, + = light, ++ = moderate, +++ = heavy.
For other tests, + = positive, 0 = negative, — = no growth.
For fermentation, numbers = pH.
Ac. = acid Cl. = clot.
* Difco thioglycollate broth. † Aerobic.

strains were isolated. One (K110) produced black pigment and apparently belongs with the ill-defined species *Bacteroides nigrescens*.[14] It was found on blood plates in intimate association with one of the streptococci (JS9), from which it was separated with difficulty. Growth occurred in Difco thioglycollate broth only when 20% of a sterile Seitz filtrate of 5-day broth culture of JS9 was added. It was later found that filtrate of a hemolytic *Micrococcus aureus* was more effective than filtrate of JS9. Biochemical activity was not tested because of alteration of the medium by the added filtrate. One strain of gram-negative nonmotile rods (JR6) grew poorly in Difco thioglycollate broth and not at all in other mediums. Its biochemical activity could not be tested except for indol and hydrogen sulfide production; both tests were negative. Strain K92 grew well on blood agar, producing green discoloration. It fermented dextrose and produced indol.

*Gram-negative motile rods.*—Of the two strains, one (JV5) was identified as *Vibrio sputorum* and has been described in detail elsewhere.[10] The second (K80) was a straight rod, slugggishly motile, and with a tendency in darkfield preparations to appear in rosette formation. It may be related to the organism described by Tunnicliff and Jackson as *Vibriothrix tonsillaris*,[15] and by Mendel as *Leptothrix asteroide*.[16]

*Fusiform rods.*—Fusiform rods were generally inconspicuous in guinea pig exudate and only one strain was isolated. It corresponds to *Fusobacterium* as described by Bøe.[17]

14. Schwabacher, H., Lucas, D. R., Rimington, C. 1947, J. Gen. Microbiol. **1**: 109–120.
15. Tunnicliff, R. and Jackson, B. L. 1930, J. Infect. Dis. **46**: 12–17.
16. Mendel, J. 1918, Compt. rend. Soc. de biol. **81**: 471–475.
17. Bøe, J. 1941, Fusobacterium Oslo Rommesjon Hos Jacob Dybwad.

*Gram-positive nonmotile rods.*—One strain (JR3) was one of the two aerobic organisms isolated. It usually formed smooth, opaque and creamy-colored colonies on blood agar but occasionally formed rough colonies. The other two strains were separated with difficulty. One (JB3A) may belong with the cocci rather than the rods. Generally it appeared as short rods, but occasionally pairs and short chains were found.

## II. RECOMBINATION OF STRAINS

### *Procedure*

Nine attempts were made to recombine all 16 bacterial strains from HG plus a bacteria-free culture of spirochetes from HG. The experimental design was essentially the same for all nine trials, with modifications in individual trials as noted below.

All cultures (except spirochetes) were grown initially from ampules stored in carbon dioxide ice. They were inoculated into Difco thioglycollate broth and as streaks on Proske-Sayers (PS) blood agar, and incubated anaerobically for 7 days, i.e., the incubation period required for the slowest growers. The growth was then recorded for both tubes and plates, and all cultures were examined microscopically. Where growth in broth was satisfactory, broth cultures were used for recombination. Otherwise growth from blood agar was used. If growth failed in both mediums, an ampule was removed from carbon dioxide ice storage and used for the recombination.

Spirochete cultures were obtained in two ways. They were grown in PSAFK agar in Noguchi tubes, using as source either spirochetes previously separated from bacteria and stored in carbon dioxide ice, or the spirochete haze surrounding confluent bacterial growth on PS blood plates that had been inoculated with HG guinea pig exudate. Alternatively, the spirochete haze from such PS blood plates was emulsified with PSAFKE broth and used directly for the recombination studies. In the latter instances, some of the emulsion was inoculated into spirochete tubes of PSAFK agar to confirm viability and as streaks on blood agar plates for both aerobic and anaerobic incubation to confirm purity. Spirochetes from the first source (separated spirochete growth in Noguchi tubes) were used in the 1st, 4th, 5th, 6th and 8th trials. Emulsified spirochetes from blood plates were used for the 2nd, 3rd, 7th and 9th trials.

Cultures were recombined on PS blood agar plates. A drop of PSAFKE broth was placed in the center of each plate. Using a wire loop, each pure culture was inoculated in turn onto the plate as a single streak *from the edge of the plate into the drop of broth in the center of the plate.* The order of inoculation of the various strains was the same on all plates. When all 16 cultures had been inoculated, each streak appeared as the spoke of a wheel with the central drop in which all cultures were merged forming the hub. Spirochetes were inoculated directly into the central drop without being streaked.

Each seed culture was, in addition, subcultured to Difco thioglycollate broth, and to blood agar plates for aerobic and anaerobic cultivation to confirm purity. Thawed guinea pig exudate from the 11th guinea pig passage was inoculated as unstreaked drops on PS blood agar and was streaked on blood agar for aerobic incubation.

Except for the aerobic blood plates, all cultures, including the recombination plates, were inoculated anaerobically in modified Brewer jars for 7 days. The aerobic plates were incubated for 3 days.

The recombination plates were examined after 7 days and the growth of each strain along its individual "spoke" recorded (fig. 1). In most instances heavy growth was found along the length of the streak and into the central drop. In some instances growth stopped short of the central drop. Sometimes a strain failed to show growth. Where all strains grew along the whole length of the streak, the agar containing the mixed growth in the central drop was cut out aseptically and emulsified in a tissue grinder with PSAFKE broth, approximately 1 ml of broth for each culture. After darkfield examination the emulsion was inoculated as unstreaked drops on fresh PS blood agar (for anaerobic incubation) and streaked on blood agar for aerobic incubation. The mixed growth of guinea pig exudate was similarly emulsified and subcultured.

After 7 days incubation the recombination drops of mixed growth on blood agar were examined grossly, emulsified in a tissue grinder as before and examined by darkfield. In those trials where growth appeared comparable grossly, and by darkfield examination, to the mixed growth of guinea pig exudate on blood agar, and where there was no evidence of contamination, the emulsified growth was inoculated into guinea pigs. The mixed growth of guinea pig exudate was inoculated into guinea pigs as a control.

### *Results*

Growth of all 16 bacterial strains and spirochetes without contamination was obtained only twice, in the 8th and 9th trials. In trial 3 all strains grew (including spirochetes), except the biochemically inactive anaerobic streptococcus, K100. Other trials were unsuccessful because of failure of certain strains to grow or because of contamination.

In the 3rd, 8th and 9th trials the growth of emulsified recombination drops exhibited a well-defined spirochete haze, ranging in diameter from 5 to 15 mm. The bacterial growth was dark brown in color and pebbled. A narrow zone (0.5 mm) of creamy, radially striated growth surrounded the central portion. Beyond this was a flat grauular pink-green iridescent margin from 0.5 to 2 mm wide. The spirochete haze was beyond this. The zone of pink and green iridescent growth, by darkfield examination, appeared to be composed solely of strain K80 (motile rods). The control cultures of guinea pig exudate appeared similar to those of the recombined cultures except that they had no iridescent margin.

## III. PATHOGENICITY OF RECOMBINED CULTURES

### *Procedure*

The mixed growth from 6 recombination drops from the 3rd, 8th and 9th trials was emulsified with PSAFKE broth (1 ml to each drop). Four normal guinea pigs were inoculated with each emulsion in a dosage of 1 ml. Inoculations were made subcutaneously into the groin without trauma. The emulsion was also streaked aerobically on blood agar. In addition, a control culture of unpurified guinea pig exudate grown on blood agar was emulsified and similarly inoculated into 4 guinea pigs. The animals were examined daily. Exudate was aspirated from appropriate lesions, examined by darkfield, and streaked on blood agar for aerobic incubation. Exudate from each of 2 animals of the experimental group was passed to 2 more animals in each series using a dosage of 0.08 ml. Exudate from 1 guinea pig in the control series was passed to 2 guinea pigs, using a dosage of 0.08 ml. Exudate from 2 of the second passage animals was passed serially

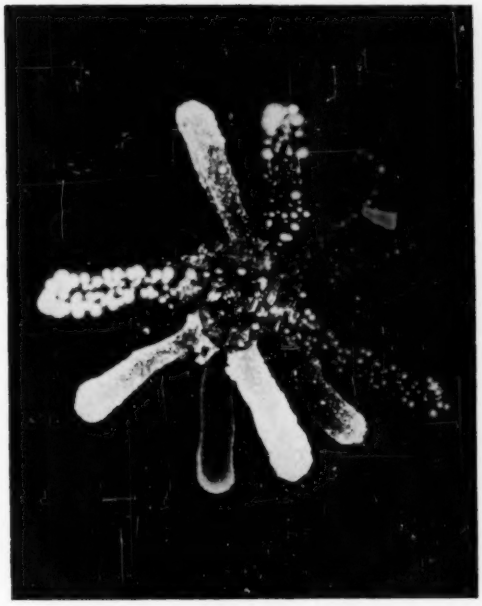

FIG. 1.—Wheel plate after 7 days anaerobic incubation, as seen by reflected light. Spirochete haze visible only by transmitted light.

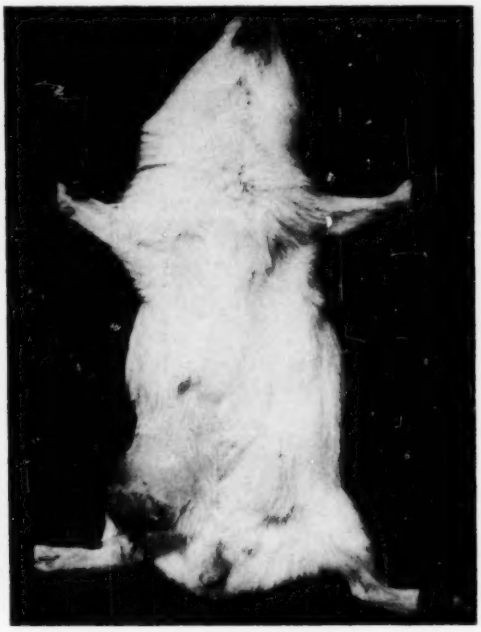

FIG. 3.—Localized abscess in experimental guinea pig (trial 8).

FIG. 2.—Localized abscess (++) in control guinea pig.

FIG. 4.—Spreading infection (+++) showing necrosis of abdominal wall in control guinea pig.

FIG. 5.—Spreading infection (+++) showing necrosis of abdominal wall in experimental guinea pig (trial 8).

through 3 more passages in all three experimental groups, using a dosage of 0.05 ml. Four animals were used at each passage in each experimental group. Exudate from only one animal in the control series was passed, except as noted in table 2. The exudate on each occasion was examined by darkfield and streaked on blood agar for aerobic incubation. All guinea pigs were autopsied when found dead or sacrificed and autopsied not later than 8 days following inoculation.

To determine the pathogenicity of each morphological group (table 1), a recombination of the organisms within each group was inoculated into guinea pigs. Recombination mixtures of the following groups were prepared according to the "wheelplate" technique detailed above: (1) streptococci, (2) gram-negative rods (including fusiforms, strain JF5), (3) motile rods, and (4) gram-positive rods. Each recombination mixture was emulsified and inoculated subcutaneously in 1 ml dosage into 2 guinea pigs. An emulsion of spirochetes grown in Noguchi tubes was similarly inoculated. No lesions developed in any of these animals, and it was therefore not possible to aspirate and pass exudate.

### *Results*

The results are recorded in table 2. Lesions developed in every guinea pig in all five passages and in all three experimental groups. The lesions were in every respect comparable to earlier descriptions of experimental fusospirochetal in-

TABLE 2.—*Pathogenicity of recombined cultures in guinea pigs.*

| Passage no. | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| Inoculum (in ml) | 1.0 cult. mash | 0.08 exudate | 0.05 exudate | 0.05 exudate | 0.05 exudate |
| Trial 3 | + P<br>+ P<br>+<br>+ | ++<br>+++ P<br>+++ P<br>++++ | +<br>+<br>+ P<br>++ P | ±<br>+ P<br>+++ P<br>+++ | +<br>++<br>+++<br>+++ |
| Trial 8 | +<br>+<br>+ P<br>++ P | + P<br>+ P<br>+<br>+ | ++ P<br>+++ P<br>+++<br>++++ | ±<br>+<br>+<br>+++ P | +<br>+<br>++<br>++ |
| Trial 9 | + P<br>++ P<br>++<br>+++ | +<br>+<br>+ P<br>+++ P | +++<br>+++<br>+++<br>+++ P | +++<br>+++<br>+++ P<br>+++ P | +<br>+<br>+<br>++++ |
| Control | + P<br>++<br>++++<br>++++ | +<br>++++ P | ++++*<br>++++*<br>++++<br>N.S.† | + P<br>++++ | ++++<br>++++ |

±, small nodular lesion. No exudate.
+, less than 1 inch localized abscess.
++, localized abscess 1 inch or more in diameter.
+++, localized abscess with extension not fatal in 7 days.
++++, spreading infection fatal in 7 days or less.
P, used for passage.
Control inoculum prepared from mixed culture of unpurified exudate.
* Animals died before exudate could be aspirated.
† Nonspecific death in 1 day. No evidence of infection.

fections in guinea pigs (fig. 3 and 5).[18] Localized lesions varied in approximate diameter from 0.5 to 5 cm. Only three lesions were nodular, without exudate (±). The larger localized lesions (+ or ++) were well walled-off abscesses containing abundant thin to thick foul-smelling exudate. The color varied from a creamy yellow to a dirty grey-brown. The walls of these abscesses showed extensive necrosis. Darkfield examination of the exudate revealed atypical fusospirochetal flora, including actively motile spirochetes of the *T. microdentium* variety, motile rods like strains JV5 and K80, nonmotile rods, fusiform rods and cocci. Lesions of this type were found in 12 of the guinea pigs from trial 3, 15 from trial 8 and 9 from trial 9.

Typical spreading fusospirochetal lesions (+++ or ++++) were found in all three trials. In animals that survived for 7 days (+++) there was generally evidence of attempted localization of the lesion. The margins showed fibrotic thickening in some instances and in some there was a deep red inflammatory border, 1 to 2 cm wide. In the three instances in which extensions proved fatal in 7 days or less (++++), there was no evidence of any attempt to localize the infection. At autopsy the lesions were found to cover most of the abdominal and thoracic walls; there was extensive necrosis of tissue and the exudate was thin and grey-brown in color. The animals lost weight rapidly during the day before death. Necrosis of skin with alopecia occurred over the abdomen of these animals and some animals in the group which showed nonfatal extensions.

Lesions in the control group of guinea pigs which were inoculated initially with a mixed culture of unpurified HG exudate were comparable to those in the experimental groups (fig. 2 and 4) but as a group were more severe. Nine of these guinea pigs developed spreading infections which were fatal in 7 days or less, and only 4 developed localized nonfatal abscesses.

18. Rosebury, T. and Foley, G. 1939, J.A.D.A. **26**: 1798–1811.

## DISCUSSION

Methods used in screening the pure cultures isolated from the HG exudate were similar to those used by Rosebury, Clark, Macdonald and O'Connell[6] in analyzing a different exudate (MP). It seems worthwhile to compare the results of the two analyses. The total number of distinctive strains isolated from each exudate was the same (16 from both, exclusive of spirochetes), and the morphologic categories were comparable. However, organisms were isolated from each exudate which clearly had no counterpart in the other exudate. Examples include the strain of *Bacteroides nigrescens* (K110) and the motile gram-negative rod, K80, from the HG exudate, and a strain presumed to be *Spirillum sputigenum* (VB7A) from the MP exudate.

These differences make it necesary to conclude that all the organisms which persist in serially transmitted fusospirochetal infections in guinea pigs are not essential to the infection or else that these comparable infections from different sources are caused by different groups of organisms, conclusions which are not mutually exclusive. In the present study K100 appears not to have been essential since pathogenicity in trial 3, from which it was missing, was comparable to that in trials 8 and 9. Further assessment of the role of individual organisms must await further study.

The difficulty experienced in attempts

to recombine the cultures isolated from HG lend support to the hypothesis that earlier attempts to infect guinea pigs with recombined cultures failed because some of the recombined cultures failed to grow in the recombined mixture. Growth of all cultures appeared in only two of the nine recombination trials. In one other (trial 3) only one strain failed to grow (K100). This particular strain failed to grow in several of the trials. Spirochetes failed in only two of the nine trials. With the exception of strain K100, no special problem was presented in obtaining growth of any of the strains. Failures occurred in an apparently haphazard fashion, suggesting that the difficulty was that of obtaining adequate growth simultaneously of a large number of fastidious strains.

The experimental infection fulfilled the requirements for fusospirochetal infection, including necrosis of tissue, production of foul-smelling exudate, typical fusospirochetal flora by darkfield examination and serial transmissibility from animal to animal. It is worth noting that the recombination mixtures used to produce infection in guinea pigs contained, among other organisms, forms that may correspond to the inadequately defined fusospirochetal quartet which Smith[2] and Proske and Sayers[3] reported as essential for the production of experimental infections, i.e., a small fusiform bacillus, a small vibrio, a streptococcus, and a thin, tightly wound spirochete—all anaerobes. The present successful production of these infections by a reproducible technique opens the way to further studies aimed at identifying the significant organisms in these complex mixtures.

## SUMMARY

1. The exudate from a serially passed guinea pig fusospirochetal infection has been analyzed into 16 pure cultures of bacteria and a culture of spirochetes.

2. These 17 cultures were recombined and cultivated together by a new technique, the "wheel plate."

3. The recombined cultures in 3 out of 9 trials, in which 16 or all 17 cultures gave evidence of separate as well as mixed growth, were successfully used to produce typical transmissible fusospirochetal infections in guinea pigs.

# MECHANISMS OF CITRATE INHIBITION OF PHAGOCYTOSIS AND OPSONIZATION

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It has long been known that citrate inhibits phagocytosis, but the mechanism is not well defined. Hektoen and Ruediger[1] treated bacteria with serum containing citrate, then washed the bacteria free from citrated serum. The presence of citrate interfered with opsonization. Leucocytes similarly treated with citrated serum, washed, and then exposed to opsonized bacteria showed no decrease in phagocytosis. The authors' conclusion[1] that citrate affected opsonization and not phagocytosis was not justified, since citrate was present during opsonization but not during phagocytosis. Studies especially concerned with brucellas by Huddleson, Johnson and Hamaan,[2] Casteneda, Tovar and Velez,[3] and Jersild[4] demonstrated that the inhibition of phagocytosis by citrate was proportional to its concentration. Maaløe,[5] without direct evidence, suggested that the citrate effect on phagocytosis was due to its anticomplementary action. The reversal of citrate inhibition of complement by $Mg^{++}$ and $Ca^{++}$ ions described by Mayer, Osler, Bier, and Heidelberger[6] suggested a possible mechanism for citrate inhibition of opsonization. The present experiments investigated citrate action on phagocytosis and opsonization, separately and combined, and its reversal by divalent cations.

## METHODS

Cells from dog blood containing 1 mg heparin per 10 ml were separated from plasma and washed 3 times, each in 10 volumes of 0.9% NaCl containing 0.3% polyethylene glycol to prevent hemolysis. Polyethylene glycol solution was free of calcium and the NaCl contained a maximum of 0.005% of Ca, Mg and $NH_3$. The solution is referred to as Na-P solution. *Brucella suis* grown on tryptose agar for 24 hours and killed by treatment with 1% formaldehyde for 24 hours were suspended at a concentration of $1 \times 10^{10}$ brucellas per ml in Na-P solution after 3 washings each in about 1000 volumes of this solution. Sodium citrate, concentration checked by drying to constant weight, and the chlorides of Ba, Ca, Cd, Co, Cu, Mg, Mn, Ni, Sr, and Zn, checked by chloride determination, were prepared in 0.774 *M* solutions. $HgCl_2$ was prepared in 0.193 *M* solution because of its low solubility. The final concentration of all divalent chlorides in the experiments was 0.053 *M*, unless stated otherwise. The final citrate concentration when employed with divalent cations was also 0.053 *M*, but when tested without divalent salts was 0.057 *M*, similar to that employed by Huddleson and others[2,3] in the opsonocytophagic test.

The effect of citrate on phagocytosis and its neutralization by divalent salts was determined by comparing phagocytosis of opsonized brucellas by washed blood cells in an equal volume of Na-P solution, in Na-P solution containing citrate, and in Na-P solution containing citrate plus divalent salt. Bacteria were opsonized by treatment with plasma during centrifugation for 30 minutes and then washed 3 times with Na-P solution. Sodium citrate, 0.2 ml of 0.774 *M*, was added to 2.5 ml of Na-P solution. Divalent salts

Received for publication February 15, 1954.

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6. Mayer, M. M., Osler, A. G., Bier O. G. and Heidelberger, M. 1946. J. Exper Med. **84**: 535.

TABLE 1.—*Citrate inhibition of phagocytosis and opsonization, separately and combined, and its neutralization by divalent cations.*

| Suspension liquid: | Control | Phagocytosis | | Opsonization | | Phagocytosis and opsonization | |
|---|---|---|---|---|---|---|---|
| | Na-P | Na-P citrate | Na-P citrate cation | Na-P | Na-P | Na-P citrate | Na-P citrate cation |
| Bacterial treatment: | Plasma | Plasma | Plasma | Plasma citrate | Plasma citrate cation | Plasma citrate | Plasma citrate cation |
| Salts 0.053*M* | Mean percentage of neutrophils phagocytosing brucellas | | | | | | |
| $MgCl_2$ | 100 | 48 | 98 | 92 | 100 | 18 | 100 |
| | 96 | 46 | 100 | 94 | 100 | 38 | 94 |
| | 100 | 94 | 100 | 70 | 84 | 24 | 72 |
| | 100 | 88 | 100 | 58 | 94 | 52 | 92 |
| $MnCl_2$ | 100 | 48 | 94 | 92 | 100 | 46 | 88 |
| | 100 | 32 | 98 | 96 | 100 | 18 | 82 |
| $NiCl_2$ | 100 | 74 | 100 | 78 | 74 | 36 | 56 |
| | 98 | 88 | 100 | 96 | [illegible] | 18 | 100 |
| $CoCl_2$ | 100 | 74 | 98 | 78 | 100 | 46 | 58 |
| $ZnCl_2$ | 96 | 46 | 92 | 94 | 86 | 32 | 20 |
| | 98 | 88 | 100 | 96 | 94 | 36 | 54 |
| $CdCl_2$ | 100 | 76 | 98 | 98 | 54 | 18 | 31 |
| | 100 | 32 | 90 | 96 | 92 | 32 | 54 |
| $CaCl_2$ | 100 | 48 | 74 | 92 | 96 | 46 | 46 |
| $BaCl_2$ | 100 | 76 | 88 | 98 | 96 | 32 | 32 |
| $SrCl_2$ | 100 | 76 | 90 | 98 | 88 | 38 | 36 |
| | 100 | 32 | 75 | 96 | 100 | 18 | 30 |
| $CuCl_2$ | 100 | 74 | 78 | 78 | 66 | | |
| | 98 | | | 96 | 96 | 18 | 36 |
| $HgCl_2$* | 96 | 46 | 70 | 94 | 24 | 38 | 40 |

* 0.0133*M* was one-fourth molar equivalent of the citrate.

were added to the Na-P citrate solution in amounts indicated in the tables. If a precipitate formed after addition of the divalent chlorides to the Na-P citrate solution, the mixture was centrifuged and the supernatant was used. To these mixtures were added equal volumes of washed blood cells. Phagocytosis was measured with a method previously described[7] by mixing 0.1 ml of bacterial suspension containing $1 \times 10^9$ formalin-killed brucellas in Na-P solution and 0.1 ml blood cell suspension. All blood cell-bacterial mixtures were rotated at 37 C for 30 minutes. Tallies were made of the percentage of 50 neutrophiles containing bacteria.

The effect of citrate on opsonization and its neutralization by divalent salts was determined by adding 0.2 ml of sodium citrate solution to 2.5 ml heparinized plasma with or without divalent chlorides. Opsonization of $10^{10}$ formalin-killed *Br. suis* was performed with 1 ml of the above solutions for 30 minutes during centrifugation at room temperature. The bacteria were then washed 3 times in Na-P solution. The degree of opsonization was determined by measuring phagocytosis of the treated bacteria by washed blood cells suspended in Na-P solutions. These experiments distinguished between the action of citrate on phagocytosis and opsonization.

Effects of citrate and divalent cations acting simultaneously on opsonization and phagocytosis were studied. Bacteria were suspended in citrate-treated and citrate-cation-treated plasmas, washed, and tested with blood cells suspended in Na-P containing citrate or citrate plus cation.

## RESULTS

*Effects of citrate on separate and combined phagocytosis and neutralization of effects by divalent cations.*—The data in table 1 demonstrate that phagocytosis of bacteria was inhibited more when 0.057 *M* citrate was present during the process of phagocytosis than when present during opsonization. The inhibitory effect of citrate acting simultaneously on phagocytosis and opsonization was much greater than the sum of the separate effects, as estimated from the percent of neutrophils phagocytosing bacteria. When paired differences were taken as a series, the mean of the control as compared with effect of citrate on phagocytosis was $37 \pm 3.3$, while the mean difference with both phagocytosis and opsonization was $71 \pm 2.6$.

7. Victor, J., Pollack, A. D., Raymond, R. and Valliant, J. R. 1952, J. Bact. **64**: 121.

Citrate inhibition of phagocytosis was partially reversed by equimolar amounts of the following divalent cations: $Mg^{++}$, $Mn^{++}$, $Ni^{++}$, $Co^{++}$, $Zn^{++}$, $Cd^{++}$, $Ca^{++}$, $Ba^{++}$, and $Sr^{++}$. $Cu^{++}$ had no effect in a single instance. $HgCl_2$ partially reversed the action of citrate when present as one-fourth the molar equivalent.

Reversal of the citrate inhibition of opsonization was demonstrable only with $Mg^{++}$, $Mn^{++}$, and $Co^{++}$, using equimolar quantities. However, reversal of combined citrate action on phagocytosis and opsonization occurred with equimolar amounts of $Mg^{++}$, $Mn^{++}$, $Ca^{++}$, $Ba^{++}$ (table 2), $Ni^{++}$, $Co^{++}$, $Cd^{++}$, and possibly $Cu^{++}$, but not with $Sr^{++}$, $Zn^{++}$, or $Hg^{++}$.

*Reversal of citrate inhibition of phagocytosis and opsonization by different concentrations of divalent salts.*—Reversal of citrate action on phagocytosis by $MgCl_2$ was complete when molarities were between one-half and equal to that of citrate, but incomplete when molarity was one-fourth that of citrate (table 2). Citrate inhibition of opsonization was reversed most effectively with equimolar $MgCl_2$, and somewhat less with lower concentrations. Reversal of citrate inhibition of combined phagocytosis and opsonization was incomplete with equimolar $MgCl_2$ and absent with one-fourth equimolar $MgCl_2$, although the latter amount had almost completely reversed the citrate effect on phagocytosis alone.

Equimolar $CaCl_2$ incompletely reversed citrate inhibition of phagocytosis, but the effect was proportional to $CaCl_2$ concentration. No evidence for citrate inhibition of opsonization was seen in the plasmas employed in the experiments with $CaCl_2$ because the plasma opsonin was too high, but citrate inhibition of combined phagocytosis and opsonization was greater than that of phagocytosis alone. Again, $CaCl_2$ only partially reversed citrate action on combined phagocytosis and opsonization when present in 0.25 to 1.0 equi-

TABLE 2.—*Citrate inhibition of phagocytosis and opsonization, separately and combined, and its neutralization by divalent cations.*

| Suspension liquid: | | Control | Phagocytosis | | Opsonization | | Phagocytosis and opsonization | |
|---|---|---|---|---|---|---|---|---|
| | | Na-P | Na-P citrate | Na-P citrate cation | Na-P | Na-P | Na-P citrate | Na-P citrate cation |
| Bacterial treatment: | | Plasma | Plasma | Plasma | Plasma citrate | Plasma citrate cation | Plasma citrate | Plasma citrate cation |
| Molarity of salt | Number of experiments | Mean percentage of neutrophils phagocytosing brucellas | | | | | | |
| $MgCl_2$ | | | | | | | | |
| 0.0534 | 4 | 99 | 69 | 99.5 | 78.5 | 94.5 | 33 | 89.5 |
| 0.0356 | 5 | 99.5 | 63.2 | 99.5 | 72 | 88.6 | 26.4 | 93.6 |
| 0.0267 | 5 | 99.5 | 63.2 | 98.5 | 72 | 85.6 | 26.4 | 75.6 |
| 0.0133 | 3 | 99.4 | 44.7 | 88.5 | 77.3 | 64 | 18.7 | 33 |
| $CaCl_2$ | | | | | | | | |
| 0.0534 | 3 | 100 | 50 | 88 | 97.5 | 98 | 17.3 | 83.3 |
| 0.0356 | 6 | 100 | 60.3 | 85.2 | 100 | 99.5 | 17 | 89.3 |
| 0.0267 | 4 | 100 | 52.5 | 70.5 | 100 | 98 | 15.5 | 84 |
| 0.0133 | 2 | 100 | 54 | 64 | 100 | 99 | 14 | 67 |
| $BaCl_2$ | | | | | | | | |
| 0.0534 | 4 | 99.5 | 70.5 | 82.5 | 97.5 | 96.5 | 28.5 | 48 |
| 0.0356 | 5 | 100 | 70.8 | 76.8 | 100 | 99.8 | 18.4 | 38.4 |
| 0.0267 | 3 | 99.3 | 68.7 | 90 | 97.3 | 95.3 | 22.7 | 19.3 |
| 0.0133 | 1 | 98 | 70 | 89 | 92 | 94 | 20 | 52 |
| $NiCl_2$ | | | | | | | | |
| 0.0534 | 6 | 99.5 | 62.4 | 94.7 | 94.4 | 84.4 | 36.4 | 75.7 |
| 0.0356 | 6 | 100 | 52 | 92.7 | 99.6 | 99.6 | 43.2 | 42.3 |
| 0.0267 | 4 | 100 | 50 | 81.5 | 99.3 | 100 | 42.7 | 31 |

molar concentrations, but reversal was similar to that with phagocytosis alone.

The highly toxic salts $BaCl_2$ and $NiCl_2$ partially reversed citrate inhibition of phagocytosis and to a lesser degree reversed citrate inhibition of combined phagocytosis and opsonization (table 2).

*Differences between citrate effects on separate and combined phagocytosis and opsonization.*—Phagocytosis of opsonized brucellas was inhibited by citrate in all of 81 tests, whereas inhibition of opsonization occurred in only 29 of 78 tests. Since there was phagocytosis by 94 to 100% neutrophiles in all the standardized tests with opsonized brucellas, all plasmas employed in the experiments contained at least 1 unit of opsonin.[7] Titrations of opsonin were not performed with the individual plasmas, but the citrate influenced their opsonizing powers to different degrees. Despite this variable effect on opsonization, citrate inhibited combined phagocytosis and opsonization to a greater degree than separate phagocytosis and opsonization in all of 81 tests.

*Differences between effects of divalent cations on reversal of citrate inhibition of separate or combined phagocytosis and opsonisation.*—Phagocytosis inhibition by citrate was reversed by all divalent cations tested except $Cu^{++}$. Opsonization inhibition by citrate was reversed only by $Mg^{++}$, $Mn^{++}$, and $Co^{++}$. Opportunities to test reversal of citrate inhibition of opsonization were limited because of the relatively high opsonin content of the plasma cited above. In additional experiments, brucellas opsonized with plasma alone or with plasma containing added 0.057 *M* $Ca^{++}$, $Mg^{++}$, or $Mn^{++}$ showed phagocytosis by 74, 76, 78 and 66% of neutrophiles, respectively. In contrast, opsonization with plasma containing $Ba^{++}$, $Cu^{++}$, $Cd^{++}$, $Co^{++}$, $Hg^{++}$, $Ni^{++}$, $Sr^{++}$ and $Zn^{++}$ showed 20, 18, 46, 54, 24, 50, 44 and 40% neutrophils phagocytosing brucellas, respectively. Thus, many cations, e.g., $Ni^{++}$, $Zn^{++}$, $Cd^{++}$, $Sr^{++}$, $Cu^{++}$, and $Hg^{++}$ added to plasma with or without citrate, inhibited opsonization when citrated plasma alone did not (table 1). The citrate combination with plasma constituents might not have been dissociated and neutralized by the cations just cited. Citrate inhibition of combined phagocytosis and opsonization was reversed most effectively by $Mg^{++}$, $Mn^{++}$, $Ca^{++}$, $Ni^{++}$, less by $Ba^{++}$, $Cd^{++}$, $Co^{++}$ and $Cu^{++}$, and not at all by $Zn^{++}$, $Sr^{++}$ and $Hg^{++}$. Reversal by all cations except $Ca^{++}$ and $Cu^{++}$ of citrate inhibition was greater with phagocytosis alone than with combined phagocytosis and opsonization. Thus, reversal of citrate inhibition by divalent cations differed in separate or combined phagocytosis and opsonization.

## DISCUSSION

Phagocytic activity of leucocytes in the preceding experiments was independent of the action of divalent cations, since the cells were washed and suspended in Na-P solutions containing only traces, i.e., less than 0.45 parts per million of Ca and Mg, of electrolytes other than sodium chloride. The inhibitory action of citrate, although reversed by divalent cations, could scarcely have been due to binding of divalent cations in Na-P solutions. Citrate must have affected the leucocytes directly, and the added divalent cations prevented the citrate from acting.

Citrate action on opsonization in the preceding experiments was limited because of relatively high opsonin activity of the plasma. The possible role of divalent cations was further tested by

comparing opsonization in plasma diluted with Na-P or Krebs polyethylene glycol solutions[7] and testing with blood cells washed 6 times in Na-P solution. Opsonin titers were measured as described elsewhere.[7] In tests with 27 plasmas showing an opsonin titer of 10 and diluted 1:100 in either of the above solutions, the mean percentage of neutrophils containing bacteria was 28.4 and 28, respectively. Employing 5 plasmas with opsonin titers of 100, tests with dilutions of 1:1000 in either Na-P or Krebs polyethylene glycol solutions showed mean percentages of neutrophils containing bacteria of 16.4 and 18, respectively. Since the maximum combined Ca and Mg concentration was 0.005% in the original NaCl crystals, then the 0.9% NaCl solution could contain 0.45 ppm of Ca and Mg. If the plasma contained 12 mg% of Ca and Mg or 120 ppm, a $10^{-2}$ dilution of plasma in Na-P solution contained 1.2 ppm of Ca and Mg in plasma plus 0.45 ppm of Ca and Mg in the Na-P solution, a total of 1.65 ppm. At a $10^{-3}$ dilution there would be 0.12 ppm plus 0.45 ppm, totalling 0.57 ppm of Ca and Mg. When combined Ca and Mg concentrations ranged between 0.57 ppm and 120 ppm there was no evidence that these cations influence opsonization. Thus the citrate effect on opsonization was probably directly on opsonin, although the added divalent cations prevented this effect.

The present observations on separate and combined phagocytosis and opsonization paralleled to a limited degree the activating effect of $Mg^{++}$ and $Ca^{++}$ on the hemolytic function of complement described by Mayer, Osler, Bier and Heidelberger.[6] Citrate inhibition of complement and its greater neutralization by $Mg^{++}$ than $Ca^{++}$ resembles that seen in phagocytosis, although complement plays no role in the ingestion of bacteria. Complement was not quantitated in the present experiments, but the slight inhibitory effect of citrate on opsonization may have been secondary to a possible effect on complement. However, the effect of citrate on complement was insignificant in the over-all effect on phagocytosis because citrate action was greatest under conditions where no complement was present, namely on the leucocytes with washed opsonized bacteria or on leucocytes with washed bacteria previously treated with citrated plasma.

The citrate effect on combined phagocytosis and opsonization was much greater than the sum of the effects on individual phagocytosis or opsonization. Thus, the combined systems appear to function with additional factors influencing bacterial ingestion. Recent studies in this laboratory demonstrated a contact phenomenon between leucocytes and bacteria enhancing phagocytosis without sensitizing bacteria or stimulating leucocytes. This contact reaction may have been inhibited by citrate.

Reversal of citrate inhibition by the divalent cations occurred with either phagocytosis or opsonization, but reversal of the combination of these functions was not predictable from the effect on either component. Combined phagocytosis and opsonization thus differs greatly from the individual components. In certain laboratories employing the opsonocytophagic test, citrate in various quantities is present during combined opsonization and phagocytosis or only during opsonization. The present experiments indicate that citrate should be avoided in all attempts to quantitate reactions between leucocytes and bacteria.

The preceding conclusions are based on statistical analyses in which mean

differences between two series were considered significant only if the probability was 0.05 or less.

## CONCLUSIONS

1. Citrate inhibition was greater in phagocytosis than opsonization. Inhibition of combined phagocytosis and opsonization was more than the sum of the effects on separate phagocytosis and opsonization. Phagocytosis and opsonization were not affected by combined Ca and Mg concentrations above 0.57 ppm. If these elements influenced these phenomena it was by action of trace amounts.

2. Reversal or neutralization of citrate inhibition by divalent cations occurred with either phagocytosis or opsonization, but reversal of the combination of these functions was not predictable from the effect on either component. Combined phagocytosis and opsonization thus differs greatly from the individual components.

# THE REVERSAL OF THE TOXICITY OF OXYTETRACYCLINE (TERRAMYCIN) BY MULTIVALENT CATIONS

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In early studies on the chemical nature of oxytetracycline, it was observed that the compound formed water-insoluble mixed salts with a number of pairs of bivalent metal ions; use was made of this property in precipitating the drug from fermentation broths.[1] Later work demonstrated that when various anions or cations were added to a simple medium, the toxicity of oxytetracycline for bacterial cells was reversed only by the multivalent cations tested: $Mg^{++}$, $Mn^{++}$, and $Fe^{++(+)}$.[2] Recently, Albert reported that oxytetracycline and chlortetracycline have an avidity for $Fe^{++(+)}$, $Cu^{++}$, $Ni^{++}$, $Co^{++}$, $Zn^{++}$, and $Mn^{++}$.[3] Also the molecular structures of the tetracycline compounds[4] reveal several enolic sites[5] at which chelation with bivalent cations might be predicted to occur.

These observations raise the following questions. (1) Is oxytetracycline toxic towards microorganisms because it competes with enzyme systems of the microbial cells for essential bivalent cations? (2) Can the active cations be employed in diagnostic work as reversing agents for the drug? (3) Will chelating agents enhance the microbial toxicity of oxytetracycline by freeing the environment of excess metallic ions? (4) If animal or microbial cells are inhibited by a toxic concentration of a metallic cation, will small quantities of the antibiotic neutralize the toxicity of the ion and thus apparently stimulate growth?

The following work was performed to extend our knowledge of the combined action of multivalent cations and oxytetracycline on bacterial growth.

## MATERIALS AND METHODS

*Bacterial strains and stock culture medium.*—Two oxytetracycline-sensitive strains of *Pseudomonas aeruginosa* were employed as the test organisms. Strain 1, with which the majority of the tests were performed, was kindly supplied by Dr. Edith Haynes, Indiana University Medical Center, Indianapolis, Indiana. Strain 2 (#9027) was obtained from the American Type Culture Collection. The cultural characteristics of each strain as determined in this laboratory agree with the description in the 6th edition of *Bergey's Manual of Determinative Bacteriology.*

The strains were serially transferred daily in a liquid medium that consisted of doubly-distilled water in which were dissolved 0.2% glucose, 0.1% $NH_4NO_3$, 0.13% $Na_2HPO_4$, 0.08% $KH_2PO_4$ 0.002965% $MgSO_4$ and 0.00006% $FeSO_4 \cdot 7H_2O$. Before inoculation the medium was adjusted to pH 7.0 with NaOH and HCl, tubed in 10 ml quantities, and autoclaved. One loopful of cells was transferred each day from the 24-hour culture to a tube of fresh sterile broth; daily plate counts during a period of 2 months demonstrated that the number of viable cells in the 24-hour culture fluctuated between $5 \times 10^8$ and $28 \times 10^8$ per ml, provided that the temperature was maintained at 37 C and that the time of incubation was precisely 24 hours. The cultures did not achieve maximum turbidity until after an incubation period of 40 hours.

*Basal medium employed for testing the sensitivity of the cells to oxytetracycline.*—The solid basal

Received for publication February 25, 1954.

It is a pleasure to acknowledge the financial support of the National Science Foundation and the excellent technical assistance of Margaret Bunton Tolzmann.

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medium employed was identical to the stock culture medium except for the addition of 2% washed agar (Difco Noble) and the omission of $MgSO_4$ and $FeSO_4 \cdot 7H_2O$. Apparently the washed agar contained trace amounts of $Mg^{++}$, $Fe^{++(+)}$, and $SO_4^{=}$ sufficient for moderate growth of the test organisms. The solid basal medium was adjusted to pH 7.0, tubed in 10 ml quantities, and autoclaved.

Sterile oxytetracycline HCl (as well as the other antibiotics used in this study) was supplied by Chas. Pfizer and Co., Inc., through the courtesy of Dr. Frederick C. Fink. The drug was dissolved and diluted in distilled water and added aseptically to tubes of the basal medium that had been liquefied and cooled to 50 C. The contents of the tubes were then thoroughly mixed and poured into sterile flat-bottomed petri plates (Pyrex 3162). The final concentrations of oxytetracycline were so adjusted that varying amounts of growth from the maximum possible on the medium through none would be obtained. The interval between each successive concentration of oxytetracycline was small; the actual quantities of the drug are presented in the section on experimental results. The drug-containing medium was inoculated immediately after the solidification of the agar by the spreading technique described below.

*Methods of adding test substances to the basal and drug-enriched medium.*—Two methods were employed to add salts of multivalent cations and other test substances to the basal and drug-containing medium; one will be called the qualitative method; the second, the quantitative method. In the former, the 24-hour broth culture was diluted in distilled water so that 10 ml contained between $5 \times 10^6$ and $28 \times 10^6$ viable cells. Of this dilution, 0.05 ml was immediately pipetted with a 0.2 ml pipette on each agar surface and spread uniformly over the surface with a flat glass spreader. A filter paper disk (#740-E; manufactured by Schleicher and Schuell, New York City) soaked in a 0.1% solution of the substance to be tested was then placed aseptically on the agar surface. The disks had been autoclaved and allowed to soak in the test solutions for 30 seconds, and had then been permitted to dry in empty sterile petri dishes. Agar surfaces containing disks soaked in distilled water as well as unsoaked disks were included in each experiment. After incubation of the inoculated mediums for 24 and 48 hours at 37 C, the diameters of the areas of stimulation or inhibition of growth around the disks were measured and the extent of the growth in the areas near the periphery of the plates was observed.

Gram stains of cells from colonies adjacent to and distant from the disks were made frequently to check for possible contamination and to ascertain the effect of the test substances and oxytetracycline on cellular morphology. Before the plates were discarded, the pH of the disks and areas adjacent to and distant from the disks was tested by the addition of a few drops of a dilute solution of brom-thymol-blue.

Qualitative tests were performed with some of the test salts in nutrient agar as well as in the basal medium previously described.

The quantitative method followed the procedure described by Foster and Pittillo.[6,7] In the present study various amounts of test solutions were added and thoroughly mixed in the liquefied, cooled basal or drug-containing medium before it was poured into petri plates. The final weight of the various cations, anions, or other substances added ranged from 0.05 to 2000 μg per ml. When large amounts of salts of weak bases and strong acids or the reverse were added, it was often necessary to readjust the pH to 7.0 with NaOH or HCl. To determine the amount of alkali or acid required, duplicate tubes of salt-enriched medium were prepared, to which were added brom-thymol-blue. The quantity of alkali or acid needed to adjust the pH to 7.0 was noted and this amount was added to the tubes actually used in the experiments.

After the neutralized salt-drug-containing medium had solidified in petri plates, cells were spread on the agar surfaces in a manner identical to that used in the qualitative method, except that the 24-hour broth culture was diluted so that 10 ml contained only between 7000 and 40,000 viable cells. Of this dilution, 0.05 ml was spread on each agar surface. After 24 and 48 hours of incubation at 37 C, the number of colonies that were present on each agar surface was counted. Duplicate control plates containing oxytetracycline and no added salts, as well as duplicate plates containing added salts but no oxytetracycline, were included in each experiment. In addition, triplicate plates of basal medium always were included.

As in the qualitative method, the morphology of the cells contained in the colonies was frequently ascertained; also noted was the size and form of the colonies, and the final pH of the colonies and surrounding medium. The final pH of medium in which no colonies appeared also was determined.

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TABLE 1.—*Test substances added to the basal medium by the qualitative (disk*) method.*

| Nitrates | Sulfates | Chlorides | Citrates | Sodium salts | Antibacterial drugs |
|---|---|---|---|---|---|
| $Na^+$ | $Na^+$ | $Na^+$ | $Na^+$ | $F^-$ | Bacitracin (2600 μg per ml) |
| $Ag^+$ | $Mg^{++}$† | $Mg^{++}$ | $Mg^{++}$ | $Cl^-$ | Chloramphenicol (60 μg per ml) |
| $Ca^{++}$† | $Mn^{++}$† | $Mn^{++}$ | | $NO_3^-$ | Chlortetracycline (60 μg per ml) |
| $Sr^{++}$ | $Fe^{++}$ † | $Fe^{++}$ | | $B_4O_7^=$ | Penicillin G (60 μg per ml)† |
| $Ba^{++}$ | $Ni^{++}$ | $Sn^{++}$† | | $So_4^=$ | Streptomycin (10 μg per ml) |
| $Co^{++}$ † | $Cu^{++}$ † | $Sb^{+++}$ | | $Se^=$ | |
| $Pb^{++}$ | $Zn^{++}$ | $Fe^{+++}$ | | $MoO_4^=$† | |
| $Hg^+$ | $Cd^{++}$ | | | Oxalate | |
| $Cr^{+++}$ | $Al^{+++}$† | | | Tartrate | |
| $Fe^{+++}$ | $Fe^{+++}$ | | | Citrate | |
| | | | | EDTA†,‡ | |

* Unless otherwise indicated, each disk was soaked in a solution of 1000 μg per ml of test substance.
† On these test substances, quantitative studies also were performed.
‡ Sodium ethylene diamine tetraacetate, kindly supplied by the Bersworth Chemical Company, Framingham, Massachusetts.

The salts and other test substances employed in the study are listed in table 1.

## EXPERIMENTAL RESULTS

*Qualitative method.*—Of the 41 test substances added to the basal medium by the qualitative method, 8 strongly reversed and 9 slightly reversed the bacterial toxicity of oxytetracycline. Ten substances slightly enchanced the toxicity of the drug and 14 substances had no effect. Of the 41 substances, 16 were toxic in the absence of the antibiotic; this toxicity was neither reversed nor enhanced by subinhibitory concentrations of oxytetracycline. The results of the qualitative tests are summarized in table 2. The sensitivity of cells of the 2 test strains to the antibiotic was nearly identical under all conditions; however,

TABLE 2.—*Effects of the test substances on the toxicity of oxytetracycline towards cells of* Ps. aeruginosa *as determined by the qualitative (disk) method.*

| (1) Test substance | (2) Factor of reversal or enhancement of test substance on toxicity of drug (see text) | (3) Area surrounding disks in presence of no or subinhibiting concentrations of drug (see text) | (4) Summary of effect of test substance on toxicity of oxytetracycline |
|---|---|---|---|
| $MgSO_4$, $MgCl_2$, $MnSO_4$, $MnCl_2$ | 12 | Neither inhibition nor stimulation around any disk. | These substances strongly reversed the toxicity of oxytetracycline. |
| $Na_2MoO_4$, Mg citrate | 6 | | |
| $FeCl_3$, $FeSO_4$ | 6 | Constant amount slight stimulation around all disks. | |
| Penicillin G | 3 | Neither inhibition nor stimulation around any disk. | These substances slightly reversed the toxicity of oxytetracycline. |
| Streptomycin, $SbCl_3$, $Ca(NO_3)_2$ | 2 | | |
| $SnCl_2$ | 3 | Constant amount slight stimulation around all disks. | |
| $FeCl_2$ | 4 | Constant zone of inhibition around all disks. | |
| $Fe_2(SO_4)_3$, $Fe_2(NO_3)_3$ | 3 | | |
| $Co(NO_3)_2$ | 2 | | |
| HOH, NaCl, NaF, $NaNO_3$, $Na_2SO_4$, $Na_2B_4O_7$, $Ba(NO_3)_2$, $Pb(NO_3)_2$, $Cr(NO_3)_3$, $ZnSO_4$ | 1 | Neither inhibition nor stimulation around any disk. | These substances had no effect on the toxicity o oxytetracycline. |
| Bacitracin, $Al_2(SO_4)_3$ Chloramphenicol, $Sr(NO_3)_2$, $Na_4EDTA$ | 1 | Constant zone of inhibition around all disks. | |
| Na tartrate, Na citrate | ½ | Neither inhibition nor stimulation around any disk. | These substances slightly enhanced the toxicity of oxytetracycline. |
| Na oxalate | ¼ | | |
| Chlortetracycline, $Hg(NO_3)_2$, $Na_2Se$ | ½ | Constant zone of inhibition around all disks. | |
| $AgNO_3$, $NiSO_4$, $CuSO_4$, $CdSO_4$ | ½ | | |

the numerical factors of reversal or enhancement listed in table 2 were calculated from data obtained with cells of strain 1. The method for calculation follows.

The concentrations of oxytetracycline in which each of the 41 substances were tested were: 0, 0.1, 0.5, 1.0, 2.0, 4.0, 6.0, 8.0 12.0, and, in a few cases, 16.0, 20.0, 24.0, 28.0, and 32.0 μg per ml of basal medium. In the control plates containing no disks, untreated disks, or disks that had been soaked in distilled water, the average maximum concentration of drug at which visible colonies appeared was 0.5 μg. per ml after 24 hours and 2.0 μg. per ml after 48 hours of incubation. In column 2 of table 2, the ability of various test substances to reverse the toxicity of oxytetracycline in the basal medium is listed in whole numbers. For example, after 48 hours at 37 C, visible colonies were present around disks soaked in $MgCl_2$ in basal medium containing as high as 24 μg per ml of drug; the factor of reversal of this salt is calculated to be 24 μg divided by 2 μg (the highest concentration that allowed growth in basal medium), or 12. (See also figure 1.)

On the other hand, if after 48 hours growth was completely inhibited at a lower concentration of oxytetracycline in the presence of a test substance than in the unenriched basal medium, the antibiotic enhancing power is calculated and listed in fractions in column 2. For example, after 48 hours the maximum concentration at which visible growth occurred in the presence of Na citrate was 1.0 μg per ml of oxytetracycline; the factor of enhancement of this salt is calculated to be 1 μg divided by 2 μg, or $\frac{1}{2}$.

In nutrient agar, the average maximum concentration of drug at which visible colonies appeared was 6.0 μg per ml after 24 hours and 8.0 μg per ml after 48 hours of incubation. This complex medium was accordingly considered to have a reversing power of 4 on the toxicity of oxytetracycline when compared with the toxicity of the drug in the simple basal medium.

Column 3 of table 2 contains information relating to the presence of a zone of inhibition that surrounded some of the disks in either basal or oxytetracycline-containing medium. It may be noted that subinhibitory concentrations of oxytetracycline (i.e., 0.1 and 0.5 μg per ml) were unable to reduce or increase the sizes of the zones of inhibition. The qualitative method, therefore, indicates that the drug at these concentrations was neither able to reverse nor to enhance the toxicity of the test substances.

*Quantitative method.*—The results of the quantitative tests usually, but not always, confirmed the less precise observations made by the qualitative method. Of great importance was the fact that, in the quantitative method, the ability of the test substances to stimulate or inhibit growth was very clearly distinguished from the effects of the test substances on the toxicity of oxytetracycline. The results obtained with strain 1 after 48 hours are presented in tables 3 to 10 and figure 2.

The end point of sensitivity to oxytetracycline in the basal medium and in each type of test substance-enriched medium was calculated to be the quantity of oxytetracycline that prevented 50% of the cells from forming visible colonies that would otherwise have done so in the basal or test substance-enriched medium in the absence of the antibiotic. This quantity of the drug is called the $ID_{50}$ rather than the $LD_{50}$ since it was not demonstrated that the inhibited cells were killed but only that their rate of reproduction was enormously slowed. In figure 2, the $ID_{50}$ values obtained with each of 7 test substances are presented.

During the 6 month period of testing,

the $ID_{50}$ in the basal medium fluctuated between 0.35 and 1.0 μg per ml; the average value was approximately 0.5 μg per ml. The small fluctuation is believed to have resulted from either small variations in the amount of active bivalent cations or other substances in various lots of wshed agar or from unknown physiological changes in the test cells during the many generations employed. The $ID_{100}$ was between 2.0 and 4.0 μg per ml, which was slightly lower than the average $ID_{100}$ of 4.0 μg per ml obtained in the qualitative method.

It may be noted in tables 3 to 10 and figure 2 that the toxicity of oxytetracycline was strongly reversed by $Fe^{++(+)}$, $Mg^{++}$, and to some extent by $Mn^{++}$ and $MoO_4^{=}$. Low concentrations of $Mn^{++}$ consistently enhanced the toxicity of the antibiotic; this phenomenon has been previously observed and reported.[2] $Co^{++}$, $Cu^{++}$, $Ca^{++}$, and penicillin G had little or no effect on the toxicity of oxytetracycline. Ethyene diamine tetra acetate and $Al^{+++}$ were inactive and are not included in the tables or figure; the very slight ability

FIG. 1.—The effect of filter paper disks soaked in $MgCl_2$ and NaCl on the antibacterial activity of oxytetracycline.

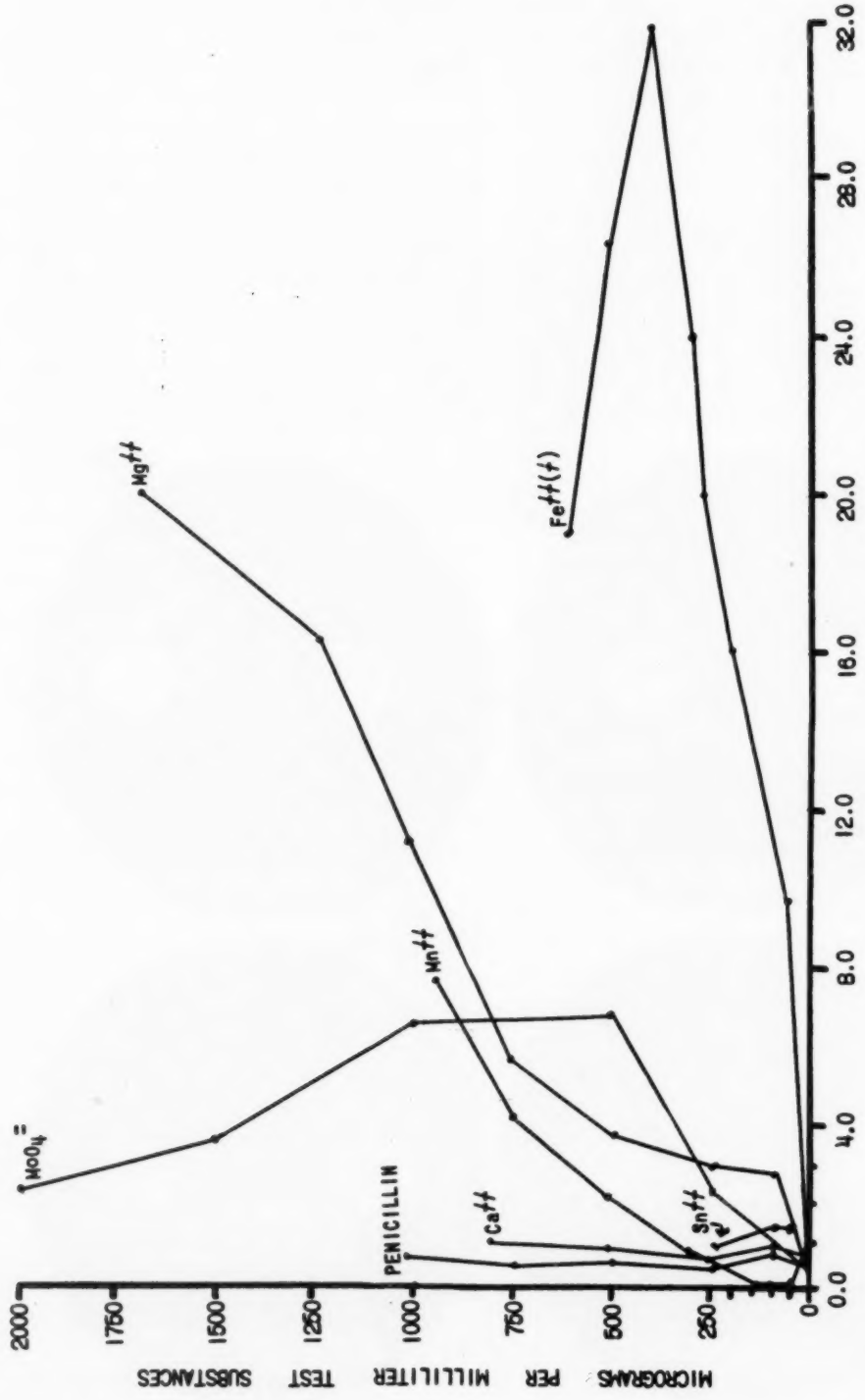


FIG. 2.—The effect of varying concentrations of 7 added test substances on the amount of oxytetracycline required to inhibit 50% of the cells of the inoculum.

of $Sn^{++}$ to reverse the toxicity of the drug is indicated in figure 2. In some of the quantitative tests (tables 3, 5, 8, and 9), slight stimulation by subinhibitory concentrations of oxytetracycline was occasionally obtained.

Reversal of toxicity of the drug by the salts of multivalent cations is believed to result from the presence of various cations rather than the respective anions ($NO_3^-$, $SO_4^=$, or $Cl^-$) because when the anions were present as sodium salts, no effect on the activity of oxytetracycline was observed when tested by the qualitative method. In quantitative tests previously reported,[2] the three anions slightly enhanced rather than reversed the toxicity of the drug at very high salt concentrations. Similarly, the effect obtained with $Na_2MoO_4$ is believed to be due to $MoO_4^=$ rather than to $Na^+$, which was inactive when present with all other anions tested.

The size of the visible colonies after 48 hours at 37 C depended to a considerable extent upon the number of colonies on each agar surface. If a surface contained 125 colonies, these were much smaller than those on a surface containing only 35 colonies. Also in the presence of high concentrations of some of the salts, colonies on both thickly and thinly populated surfaces were small. On the other hand, high concentrations of oxytetracycline either suppressed the formation of visible colonies entirely or else allowed the colonies to develop to the maximum size permissible by the other conditions cited above.

In the majority of tests smooth colonies developed, but in the presence of high concentrations of $Mg^{++}$, $Mn^{++}$, or $Fe^{++(+)}$, many of the colonies were filamentous. This observation is analogous to that made by Cole and Braun[8] with broth cultures of a strain of *Brucella abortus*. Also of interest is the fact that in the presence of high concentrations of $MgSO_4$, a precipitate (Mg phosphate?) formed which was dissolved by the reproducing cells so that a clear halo appeared around each individual colony.

Cellular morphology was quite homogeneous throughout the experiments, except in the presence of high concentrations of penicillin plus oxytetracycline, in which case may of the cells appeared longer than usual and a few filamentous cells were found. The homogeneity of cellular morphology obtained under the many environmental conditions of this study is not unexpected since only cells of visible colonies were examined. Cells that were inhibited by either oxytetracycline or the test substances could not reproduce rapidly enough to form visible colonies. Pulvertaft has performed an excellent cytological study on such cells by continuously observing agar slide cultures that contained varying amounts of oxytetracycline or other antibiotics.[9]

In most of the quantitative and qualitative tests of the present study, the buffering action of the synthetic basal medium was sufficient to maintain the pH at neutrality during incubation. In nutrient agar, growth of cells of the test strains resulted in the development of a alkaline pH. As in previous work,[2] the addition of large quantities of the sulfates of $Fe^{++(+)}$ or $Mn^{++}$ to the synthetic basal medium by the quantitative method resulted in a final slightly acid rather than a neutral pH. However, cells of the test strains were able to grow satisfactorily in the basal medium at pH 6.5 and were even more sensitive to oxytetracycline at this pH than at neutral-

8. Cole, L. J. and Braun, W. 1950, J. Bact. **60**: 283–289.

9. Pulvertaft, R. J. V. 1952, J. Path. & Bact. **64**: 75–89.

TABLE 3.—*The effect of varying concentrations of added $FeSO_4 \cdot 7H_2O$ and oxytetracycline on the number of visible colonies developing from a fixed inoculum. The figures represent the average colony counts of 6 experiments.*

| $Fe^{++(+)}$, μg per ml | Oxytetracycline, μg per ml | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0.0 | 0.1 | 1.0 | 3.0 | 4.0 | 8.0 | 12.0 | 16.0 | 20.0 | 24.0 | 32.0 |
| 700 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 600 | 68 | 73 | 64 | 74 | 74 | 77 | 64 | 73 | 22 | 12 | 0 |
| 500 | 144 | 134 | 176 | 127 | 112 | 116 | 133 | 94 | 96 | 85 | 31 |
| 400 | 128 | 149 | 200 | 126 | 103 | 88 | 121 | 81 | 72 | 75 | 64 |
| 300 | 178 | 179 | 198 | 168 | 144 | 102 | 108 | 115 | 97 | 88 | 48 |
| 200 | 187 | 197 | 181 | 144 | 148 | 126 | 108 | 96 | 65 | 84 | 48 |
| 100 | 182 | 185 | 127 | 110 | 138 | 156 | 140 | 64 | 61 | 68 | 38 |
| 50 | 200 | 214 | 161 | 174 | 138 | 131 | 80 | 80 | 48 | 54 | 13 |
| 10 | 235 | 269 | 306 | 200 | 210 | 153 | 66 | 32 | 11 | 1 | 0 |
| 5 | 252 | 264 | 155 | 96 | 40 | 13 | 18 | 5 | 0 | 0 | 0 |
| 1 | 218 | 158 | 106 | 12 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| 0 | 119 | 110 | 41 | 5 | 2 | 0 | 0 | 0 | 0 | 0 | 0 |

ity. Since the salts of $Fe^{++(+)}$ and $Mn^{++}$ reversed rather than enhanced the toxicity of the drug, the action of these salts on the antibiotic is apparently independent of their effect on the final hydrogen ion concentration.

In table 3, it may be noted that the addition of between 1 and 300 μg per ml $Fe^{++(+)}$ to the basal medium allowed from 2 to 1.5 times as many colonies to develop as in the unenriched basal medium. Large amounts of penicillin or $MoO_4^{=}$ likewise stimulated more cells to form visible colonies. Thus, the spreading technique of Foster and Pittillo[6] in these instances revealed that the basal medium was not the optimum medium for maximum growth. Other types of inoculation such as streaking or spreading thousands of cells on the agar surfaces did not reveal this fact. Foster and Pittillo[6] have noted that many complex organic supplements are found to be toxic rather than stimulatory when less than 200 cells are spread on the surface of mediums with and without the supplements. Lamanna and Mallette[10] have observed that the spread plate technique with a fixed quantity of inoculum is a very convenient method for testing and comparing various mediums.

In the present work, when small amounts of $FeSO \cdot 7H_2O$ were incorporated in the medium, a dilution of 50% was made in the inoculum so that the number of developing colonies could be conveniently and accurately counted. The number was then multiplied by 2 before being entered in table 3. The observation that the test strains of *Pseudomonas* were unusually sensitive to oxytetracycline was probably due in part to the fact that the basal medium contained suboptimal quantities of $Fe^{++(+)}$. However, even in nutrient agar which presumably contains sufficient $Fe^{++(+)}$ for maximum growth, the strains were fairly sensitive to the drug. In nutrient agar, also, the addition of more $Fe^{++(+)}$ or $Mg^{++}$ reversed the toxicity of oxytetracycline.

In table 6 the extent of stimulation of growth by penicillin G may be noted; this observation confirms the work of Garrod.[11] The mechanism of stimulation of gram-negative bacilli by penicillin remains to be elucidated.

## DISCUSSION

The phenomenon of reversal of antibiotic activity by bivalent cations has been observed with at least 2 other drugs: chlortetracycline and polymyxin. Van Meter et al[12] noted that oxidation

10. Lamanna, C. and Mallette, M. F. 1953, Basic Bacteriology, Baltimore, The Williams and Wilkins Company, pp. 383–384.

11. Garrod, L. P. 1951, Brit. M. J. **1**: 205–210.

12. Van Meter, J. C., Spector, A., Oleson, J. and Williams, J. H. 1952, Proc. Soc. Exper. Biol. & Med. **81**: 215–217.

TABLE 4.—*The effect of varying concentrations of added $MgSO_4$ and oxytetracycline on the number of visible colonies developing from a fixed inoculum. The figures represent the average colony counts of 5 experiments.*

| $Mg^{++}$, μg per ml | Oxytetracycline, μg per ml | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 0.0 | 0.1 | 0.5 | 1.0 | 3.0 | 6.0 | 9.0 | 12.0 | 15.0 | 18.0 |
| 1800 | 0 | | | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 1650 | 65 | | | 78 | | 46 | 42 | 57 | 55 | 47 |
| 1500 | 62 | | | 82 | 54 | 43 | 57 | 62 | 42 | 23 |
| 1350 | 78 | | | 80 | 58 | 55 | 64 | 49 | 42 | 35 |
| 1200 | 75 | | | 71 | 86 | 58 | 46 | 54 | 34 | 38 |
| 1050 | 94 | | | 93 | 100 | 82 | 85 | 77 | 46 | 34 |
| 900 | 77 | 77 | 82 | 101 | 103 | 74 | 39 | 45 | 42 | 26 |
| 750 | 96 | 81 | 94 | 70 | 65 | 48 | 37 | 43 | 21 | 10 |
| 600 | 83 | 91 | 80 | 65 | 46 | 27 | 26 | 22 | 23 | 8 |
| 450 | 81 | 69 | 64 | 57 | 43 | 31 | 26 | 23 | 25 | 21 |
| 300 | 95 | 77 | 75 | 64 | 48 | 41 | | 21 | 25 | 15 |
| 150 | 100 | 129 | 128 | 92 | 49 | 41 | | 23 | | |
| 100 | 100 | 99 | 119 | 93 | 48 | 37 | 10 | 12 | 0 | 0 |
| 50 | 120 | 112 | 99 | 53 | 30 | 19 | 8 | 0 | 0 | 0 |
| 0 | 114 | 104 | 53 | 40 | 6 | 0 | 0 | 0 | 0 | 0 |

by kidney and liver mitochondria was inhibitied by chlortetracycline in the absence but not in the presence of $Mg^{++}$; it was concluded that the drug and metal combined to form an insoluble salt. Saz and Slie[13] observed that the inhibition by chlortetracycline of a bacterial cell-free nitro-reductase was reversed by the addition of $Mn^{++}$, and postulated that the drug inhibited the enzymatic system by chelating this cation, which is essential to the system. The reversal of various antibacterial activities of chlortetracycline and oxytetracycline by $MgCl_2$ has also been reported by Soncin.[14]

Newton[15] has recently demonstrated that $Mg^{++}$, $Ca^{++}$, $Mn^{++}$, and $Fe^{++(+)}$ reversed the ability of polymyxin to inhibit growth of *Ps. aeruginosa*. He found that 400 atoms of $Mg^{++}$ reversed 1 molecule of polymxin and concluded that the nontoxic cations compete with polymyxin for absorption sites on or in the cells rather than that the ions form a complex with the drug.

In the present study, the number of atoms of $Mg^{++}$ that reversed 1 molecule of oxytetracycline varied with the concentration of $Mg^{++}$; in the range between 300 and 1500 μg per ml of $Mg^{++}$, approximately 2000 atoms of $Mg^{++}$ were required to reverse 1 molecule of the drug. $Fe^{++(+)}$ was more efficient: in the range between 50 and 400 μg per ml of $Fe^{++(+)}$, each molecule of oxytetracycline was neutralized by between 47 and 141 atoms of $Fe^{++(+)}$. The most efficient concentration of $Fe^{++(+)}$ was 50 μg per ml.

13. Saz, A. K., and Slie, R. B. 1953, J. Am. Chem. Soc. **75**: 4626.
14. Soncin, E. 1953, Arch. Int. Pharmacodyn. **94**: 346–352.
15. Newton, B. A. 1953, Nature, **172**: 160–161.

TABLE 5.—*The effect of varying concentrations of added $MnSO_4 \cdot H_2O$ and oxytetracycline on the number of visible colonies developing from a fixed inoculum. The figures represent the average colony counts of 5 experiments.*

| $Mn^{++}$, μg per ml | Oxytetracycline, μg per ml | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 0.0 | 0.1 | 0.5 | 1.0 | 2.0 | 3.0 | 4.0 | 8.0 | 12.0 | 16.0 |
| 1000 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 900 | 63 | 56 | 66 | 72 | 67 | 60 | 48 | 30 | 18 | 0 |
| 750 | 78 | 62 | 78 | 76 | 54 | 67 | 41 | 22 | 21 | 22 |
| 600 | 85 | 65 | 75 | 77 | 72 | 37 | 21 | 12 | 10 | 0 |
| 450 | 78 | 67 | 85 | 54 | 7 | 7 | 0 | 0 | 0 | 0 |
| 300 | 61 | 52 | 57 | 44 | 2 | 0 | 0 | 0 | 0 | 0 |
| 150 | 75 | 15 | 10 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 50 | 74 | 18 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 10 | 105 | 147 | 126 | 15 | 0 | 0 | 0 | 0 | 0 | 0 |
| 5 | 120 | 165 | 135 | 56 | 0 | 0 | 0 | 0 | 0 | 0 |
| 0 | 151 | 132 | 92 | 61 | 18 | 10 | 6 | 0 | 0 | 0 |

TABLE 6.—*The effect of varying concentrations of added penicillin G and oxytetracycline on the number of visible colonies developing from a fixed inoculum. The figures represent the average colony counts of 3 experiments.*

| Penicillin G, μg per ml | Oxytetracycline, μg per ml | | | | |
|---|---|---|---|---|---|
| | 0.0 | 0.5 | 1.0 | 3.0 | 4.0 |
| 1000 | 32 | 17 | 10 | 4 | 0 |
| 800 | 101 | 48 | 3 | 0 | 0 |
| 600 | 86 | 38 | 10 | 6 | 0 |
| 400 | 102 | 42 | 23 | 7 | 0 |
| 200 | 121 | 49 | 22 | 3 | 0 |
| 100 | 120 | 64 | 30 | 13 | 0 |
| 0 | 81 | 40 | 19 | 3 | 0 |

TABLE 8.—*The effect of varying concentrations of added $Na_2MoO_4 \cdot 2H_2O$ and oxytetracycline on the number of visible colonies developing from a fixed inoculum. The figures represent the average colony counts of 3 experiments.*

| $MoO_4^{=}$, μg per ml | Oxytetracycline, μg per ml | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 0.0 | 0.1 | 0.5 | 1.0 | 4.0 | 8.0 | 12.0 | 16.0 |
| 2000 | 55 | 72 | 67 | 33 | 22 | 10 | 9 | 0 |
| 1500 | 98 | 82 | 86 | 81 | 49 | 17 | 27 | 26 |
| 1000 | 111 | 108 | 114 | 108 | 97 | 25 | 23 | 4 |
| 500 | 157 | 184 | 140 | 116 | 121 | 34 | 0 | 0 |
| 250 | 109 | 138 | 108 | 85 | 29 | 0 | 0 | 0 |
| 0 | 90 | 70 | 21 | 6 | 0 | 0 | 0 | 0 |

No conclusion concerning the mechanism of metal reversal of the toxicity of oxytetracycline should be made at this time because certain information is lacking concerning the phenomenon. For example, the extent of diffusion into the cells of pure drug versus the drug-metal complex (if any) should be ascertained. The extent of absorption of oxytetracycline on the cell surface and on enzymes within the cell should be determined. Helpful additional information would be the precise number of atoms of $Fe^{++(+)}$ required for a single normal cell division and the number of molecules of drug required to inhibit a single normal cell division. A comparison of the effects of the tetracycline compounds on many isolated bacterial enzyme systems that require specific metals with the effects on enzyme systems that need no metals must also be made.

In the present work, no evidence of counteraction by oxytetracycline of the toxicity due to metals was observed in the qualitative tests. In the quantitative work, the presence of 0.1, 0.5, or 1.0 μg per ml of the drug occasionally resulted in the appearance of slightly more visible colonies than in the comparable drug-free metal-enriched control medium (tables 3, 5, 8, and 9).

With respect to the kinds of multivalent cations that could reverse the toxicity of oxytetracycline, it should be emphasized that the present results were obtained by microbiological rather than chemical methods of assay. The requirements of cells of *Pseudomonas* for a spectrum of metallic ions in definite concentrations must certainly have influenced the observations. For example, Regna et al[1] reported that salts of $Ca^{++}$ and $Ba^{++}$ combined with oxytetracycline to form insoluble mixed salts. Also, Albert[3] found that $Zn^{++}$ and $Ni^{++}$ combined with the antibiotic. In the present

TABLE 7.—*The effect of varying concentrations of added $Ca(NO_3)_2 \cdot 4H_2O$ and oxytetracycline on the number of visible colonies developing from a fixed inoculum. The figures represent the average colony counts of 3 experiments.*

| $Ca^{++}$, μg per ml | Oxytetracycline, μg per ml | | | | |
|---|---|---|---|---|---|
| | 0.0 | 0.1 | 0.5 | 1.0 | 3.0 |
| 1000 | 0 | 0 | 0 | 0 | 0 |
| 800 | 11 | 12 | 8 | 0 | 0 |
| 600 | 48 | 24 | 24 | 9 | 0 |
| 400 | 41 | 24 | 25 | 0 | 6 |
| 200 | 60 | 43 | 24 | 15 | 0 |
| 100 | 59 | 47 | 80 | 24 | 0 |
| 50 | 85 | 67 | 48 | 13 | 0 |
| 0 | 135 | 130 | 101 | 60 | 0 |

TABLE 9.—*The effect of varying concentrations of added $Co(NO_3)_2 \cdot 6H_2O$ and oxytetracycline on the number of visible colonies developing from a fixed inoculum. The figures represent the average colony counts of 4 experiments.*

| $Co^{++}$, μg per ml | Oxytetracycline, μg per ml | | | | | |
|---|---|---|---|---|---|---|
| | 0.0 | 0.1 | 0.5 | 1.0 | 2.0 | 3.0 |
| 3.5 | 0 | 0 | 0 | 0 | 0 | 0 |
| 3.0 | 27 | 43 | 37 | 40 | 22 | 0 |
| 2.5 | 86 | 52 | 46 | 51 | 10 | 1 |
| 2.0 | 68 | 90 | 43 | 39 | 19 | 0 |
| 1.5 | 68 | 100 | 53 | 22 | 19 | 0 |
| 1.0 | 76 | 95 | 50 | 34 | 18 | 0 |
| 0.5 | 98 | 102 | 93 | 40 | 37 | 0 |
| 0.1 | 84 | 94 | 50 | 35 | 9 | 0 |
| 0.0 | 126 | 109 | 37 | 12 | 9 | 0 |

TABLE 10.—*The effect of varying concentrations of added $CuSO_4 \cdot 5H_2O$ and oxytetracycline on the number of visible colonies developing from a fixed inoculum. The figures represent the average colony counts of 3 experiments.*

| $Cu^{++}$, μg per ml | Oxytetracycline, μg per ml | | | | | |
|---|---|---|---|---|---|---|
| | 0.0 | 0.1 | 0.5 | 1.0 | 2.0 | 3.0 |
| 3.5 | 1 | 0 | 0 | 0 | 0 | 0 |
| 3.0 | 9 | 2 | 2 | 0 | 0 | 0 |
| 2.0 | 19 | 33 | | 12 | 5 | 0 |
| 1.0 | 55 | 51 | 40 | 27 | 18 | 5 |
| 0.5 | 76 | 89 | 69 | 34 | 21 | 7 |
| 0.1 | 85 | 71 | 49 | 30 | 24 | 12 |
| 0.05 | 80 | 82 | 47 | 20 | 23 | 8 |
| 0.0 | 76 | 70 | 40 | 11 | 4 | 2 |

work, however, these four ions were inactive in reversing the toxicity of the drug towards *Pseudomonas* cells. A possible explanation for their inactivity is that the drug-metal complexes are toxic but that only those metallic ions that are most essential to the enzymes of *Pseudomonas* [$Fe^{++(+)}$, $Mg^{++}$, and to some extent $Mn^{++}$ and $MoO_4^{=}$] can neutralize the toxic drug or drug-metal complexes and restore enzymatic activity.

A somewhat analagous situation has been reported by Braude et al[16] who found that oxytetracycline is almost as effective an anticoagulant as is citrate and is 10 to 12 times as effective as is streptomycin or penicillin. However, $Ca^{++}$ was unable to reverse the anticoagulating ability of oxytetracycline whereas $Ca^{++}$ did reverse the activity of the other two antibiotics.

It was also considered in the present study that the addition of a sodium salt of a metal-binding ion such as citrate, oxalate, tartrate, or ethylene diamine tetra acetate might enhance the toxicity of oxytetracycline by removing free metallic ions from the environment. However, in qualitative and quantitative tests with these salts, only slight or no enhancement of the toxicity of the drug was obtained.

16. Braude, A. I., Davey, P. and Siemienski, J. 1953, Proc. Soc. Exper. Biol. & Med. **83**: 160.

The ability of $Fe^{++(+)}$ and $Mg^{++}$ to reverse the antagonism of oxytetracycline towards cells of *Pseudomonas* was also observed when nutrient agar was employed as the basal medium. The phenomenon of metallic ion reversal of oxytetracycline and chlortetracycline also was noted when cells of a strain of colorless alga (*Prototheca chlorelloides*) were employed as the test organisms.[17] It will be of interest to extend the observation to pleuropneumonia-like organisms and to the protozoa. And since the formation of filamentous cells of gram-positive bacilli[18,19] and of long chains of gram-positive diplococci[20] is partially a function of the concentration of $Mg^{++}$, the effect of various concentrations of $Mg^{++}$ and oxytetracycline on the cellular morphology of gram-positive bacteria should be thoroughly investigated.

## SUMMARY

The mutual effects of oxytetracycline and each of 18 multivalent inorganic cations, 8 multivalent anions, and 5 antibiotics on the growth of cells of *Pseudomonas aeruginosa* was studied. The toxicity of oxytetracycline was strongly reversed by salts of $Fe^{++(+)}$, $Mg^{++}$, $Mn^{++}$, and $MoO_4^{=}$. The inclusion in the medium of subinhibiting concentrations of oxytetracycline occasionally reversed slightly the toxicity of bivalent cations. Penicillin stimulated growth of test organisms in the basal medium but had no effect on the activity of oxytetracycline. To detect mutual reversal or enhancement of the toxicities of drugs and other test substances or to detect stimulation of growth by a single drug, the spread plate technique of Foster and Pittillo is recommended.

17. Tychbrocher, M. Personal communication.
18. Shanker, K. and Bard, R. C. 1952, J. Bact. **63**: 279–290.
19. Webb, M. 1953, Science, **118**: 607–611.
20. Rochford, E. J. and Mandle, R. J. 1953, J. Bact. **66**: 554–560.

# RELATIONSHIP BETWEEN *BARTONELLA MURIS* INFECTION AND ACUTE RADIATION EFFECTS IN THE RAT

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Infection with *Bartonella muris* is endemic in many rat colonies. While it is now known that the acute infection may be prevented by chemotherapy with aureomycin and terramycin[1] or the organic arsenicals,[2] such treatment is not usually employed because the infection is dormant and the animal symptom-free unless splenectomy is performed. However, its presence may become a complicating factor in experiments with ionizing radiation because of the radiosensitivity of the spleen and possible damage to the protective mechanism. Also, the infection may play a role in the hematologic changes following irradiation since bartonellosis, in itself, produces hematuria and anemia. The experiments reported here were undertaken to ascertain (a) whether ionizing radiation from external or internal sources would indeed activate latent bartonella infection in the rat, (b) the possible effect of the infection on the severity and course of acute radiation sickness, and (c) any effect of irradiation on the course of bartonellosis. It is commonly assumed that the infection would exacerbate radiation effects.

The results indicate that both the $\alpha$ particles from polonium which have a pronounced tendency to concentrate in spleen[3,4] or whole body irradiation with 250 KV X rays can activate the disease in rats. The relative effects of these agents on the subsequent anemia and mortality differ, however, both from each other and from surgical splenectomy. Contrary to expectations, the presence of the active infection seemed to have no tendency to increase the mortality.

## MATERIALS AND METHODS

Two strains of rats were used for the polonium experiments: one the Sprague-Dawley (Madison, Wisconsin), which is normally bartonella-free and was kept so under our experimental conditions, as evidenced by splenectomy; the other the local colony (Wistar derived, but termed here the "Rochester" strain), known to carry *Haemobartonella muris* latently. The X-ray experiments employed only the Rochester rats. The infected and uninfected animals were kept in the same room but many precautions were taken to prevent cross-infection. The animals were kept in separate cages and the groups kept on separate racks whose supports were treated to prevent the

Received for publication March 12, 1954.

This paper is based on work performed under contract with the United States Atomic Energy Commission at the University of Rochester Atomic energy Project, Rochester, New York. Portions have appeared in University of Rochester Atomic Energy Project Reports UR-145 and UR-200 (limited circulation), and an abstract has been submitted for the 38th Annual Meeting of the Federation of American Societies for Experimental Biology at Atlantic City, April 1954.

* With the technical assistance of Mildred Carr.

1. Stanton, M. F., Laskowski, L. and Pinkerton, H. 1950, Proc. Soc. Exper. Biol. & Med. **74**: 705–707.
2. Farris, E. J. and Griffith, J. Q. 1949, The Rat in Laboratory Investigation, ed. 2, Philadelphia, J. B. Lippincott Co., p. 497.
3. Fink, R. M., ed. 1950, Biological Studies with Polonium, Radium, and Plutonium, New York, McGraw-Hill Book Co., Inc., pp. 35–121.
4. Finkel, M., Norris, W. P., Kisieleski, W. E. and Hirsch, G. M. 1953, Am. J. Roentgenol. **LXX**: 477–485.

access of rat lice, presumed to be a primary carrier of the bartonella infection. The food was kept in metal cans away from possible wild rodent contamination. Controls and survivors were kept for many months (usually allowed to complete their life-span) and occasional individuals splenectomized. No evidence of adventitious bartonella infection appeared in the Sprague-Dawley (uninfected) animals at any time, even though the neighboring racks contained many animals with infections.

The polonium dosages were given as the chloride by injection into the femoral vein at a level of about 40 microcuries per kg body weight. The solutions were prepared from a stock in 0.5 *N* HCl by bringing it to pH 6.5 and diluting with 0.9% saline. Because of its high specific activity there is no question of heavy metal toxicity with polonium. Also, it decays with almost pure $\alpha$-particle emission to a stable isotope of lead. As indicated above, its tendency to concentrate in spleen makes it of special interest for this study because it might be expected to produce an "autosplenectomy." Most of the animals injected were adult males, but occasional females were used.†

The X-ray doses were given to adult female rats. The dose was 600 r, measured in air, with radiation factors as follows: 250 KV, 15 ma; aluminum parabolic filter (14 mm thick in center) plus 0.5 mm copper; target to skin distance 25 inches; and rate 19.5 r/min.

Following treatment all animals in the X-ray group and selected animals (set aside for the purpose) in the polonium group were sampled for red cell counts and blood films were prepared. The smears were examined especially for the presence of *Bart. muris* organisms in the red cells. Their presence in abundance, accompanied by anemia and hematuria, was taken as indicative of an active infection. All animals in the polonium-treated group were weighed daily. This was not done in the X-irradiated animals because of the large backlog of such data on animals from this colony. Observations were made 6 days a week.

## RESULTS

Preliminary experiments were performed to determine the presence or absence and course of the bartonellosis in the two strains of rats. Fifteen animals from the local colony and fifteen Sprague-Dawley animals were splenectomized. The latter exhibited no changes other than those expected following anesthesia and the surgical procedures. After splenectomy all of the Rochester rats lost weight, had hemoglobinuria, and films of the peripheral blood showed many bartonella organisms within the red cells. The hemolytic crises were repeated and all animals developed anemia of varying degrees. All died within 2 months, the first animal at 4 days, the last at 2 months.

### *1. Polonium experiments*

The first experiment with polonium employed 60 animals, 30 Sprague-Dawley and 30 Rochester, and was performed to compare the effect of polonium on infection-free and infected animals. The lethality data are summarized in table 1 (exp. 1), expressed as the number and percent of animals dying in various time intervals after injection. The Sprague-Dawley animals received an average polonium dose of 46 $\mu$c/kg ($\pm$4.0, standard deviation) and their median survival time was 19 days. The Rochester animals received an average dose of 43.5 $\mu$c/kg ($\pm$4.1) and exhibited a median survival time of 34 days. This difference in survival time is not only much larger than would be expected from the small differences in average dose[5] but is in the opposite direction from that expected if bartonella infection exacerbated the lethal radiation effects.

The Rochester rats always showed more anemia (see table 2, exp. 1) and bartonella organisms appeared in the

† These females are not included in the toxicity data shown. However, no real or regular sex differences in the acute lethal effects of either polonium or X rays have been found in the course of many years of work in this laboratory.

5. Fink, R. M., ed. 1950, Biological Studies with Polonium, Radium, and Plutonium, New York, McGraw-Hill Book Co., Inc., pp. 211–404.

TABLE 1.—*Mortality data for rats injected with polonium (number dying during designated periods).*

| Days after injection | Sprague-Dawley | | Rochester | | Rochester with Mapharsen | |
|---|---|---|---|---|---|---|
| | Number | Percent | Number | Percent | Number | Percent |
| | | | Experiment 1 | | | |
| 0–10 | 1 | 3.3 | 0 | 0.0 | | |
| 11–20 | 14 | 46.7 | 3 | 10.0 | | |
| 21–30 | 7 | 23.3 | 10 | 33.3 | | |
| 31–40 | 5 | 16.7 | 6 | 20.0 | | |
| 41–50 | 2 | 6.7 | 6 | 20.0 | | |
| 51–60 | 0 | 0.0 | 2 | 6.7 | | |
| 61–70 | 1 | 3.3 | 1 | 3.3 | | |
| 71–90 | | | 1 | 3.3 | | |
| >90 | | | 1* | 3.3 | | |
| Total | 30 | | 30 | | | |
| Dose | 46 μc/kg (±4.0) | | 43.5 μc/kg (±4.1) | | | |
| | | | Experiment 2 | | | |
| 0–10 | 0 | 0.0 | 0 | 0.0 | 0 | 0.0 |
| 11–20 | 12 | 54.5 | 5 | 20.8 | 8 | 36.4 |
| 21–30 | 7 | 31.8 | 9 | 37.5 | 7 | 31.8 |
| 31–40 | 0 | 0.0 | 5 | 20.8 | 1 | 4.6 |
| 41–50 | 1 | 4.6 | 3 | 12.5 | 1 | 4.6 |
| 51–60 | 0 | 0.0 | 1 | 4.2 | 3 | 13.6 |
| 61–70 | 1 | 4.6 | 0 | 0.0 | 1 | 4.6 |
| 71–90 | 0 | 0.0 | 1 | 4.2 | 0 | 0.0 |
| >90 | 1† | 4.6 | | | 1‡ | 4.6 |
| Total | 22 | | 24 | | 22 | |
| Dose | 33.1 μc/kg (±1.2) | | 37.7 μc/kg (±2.7) | | 41 μc/kg (±2.4) | |

* At 106 days.
† At 92 days.
‡ At 152 days.

peripheral blood at 10 days. By 28 days all animals receiving polonium showed Bartonella organisms. The Sprague-Dawley rats showed less anemia (table 2) and no evidence of bartonella infection. Control animals of either strain showed no evidence of bartonella and no mortality in 100 days.

A second experiment was done to check and amplify these findings. In table 1 (exp. 2) mortality data are given for three additional goups of rats. Two are essentially repetitions of experiment 1 and will be discussed here. The third is taken up separately. A group of 22 Sprague-Dawley rats received an average polonium dose of 33.1 μc/kg (±1.2) and showed a median survival time of 20 days. A similar repeat group of 25 Rochester rats received an average polonium dose of 37.7 μc/kg (±2.7) and their median survival time was 27 days. Thus, the same general result was obtained as in experiment 1. The uninfected (Sprague-Dawley) animals seemed to die at least as rapidly as the Rochester group (even though their polonium dose was slightly lower). The small difference in survival times may be adventitious, and we would propose from these data (and those from experiment 1) only that the presence of the bartonella infection does not increase the toxic effects of polonium.

The data shown thus far do not distinguish between the role of infection per se and a possible strain difference in sensitivity to polonium. An approach to

TABLE 2.—*Average red cell counts of Sprague-Dawley (S-D) and Rochester (Roch) rats after polonium injection**

| Experiment 1 | | | Experiment 2 | | | Experiment 3 | | |
|---|---|---|---|---|---|---|---|---|
| Time, days | S-D, million/mm³ | Roch, million/mm³ | Time, days | Roch, million/mm³ | Roch with Mapharsen, million/mm³ | Time, days | S-D (uninfected), million/mm³ | S-D (infected), million/mm³ |
| 0 | 8.3 (16)* | 8.1 (15)* | 0 | 7.9 (16)* | 7.2 (16)* | 0 | 8.1 (27)* | 8.5 (24)* |
| 1 | 8.4 (16) | 8.0 (15) | 1–2 | 7.7 (16) | 7.7 (16) | 2 | 8.4 (28) | 8.0 (26) |
| 5 | 8.0 (16) | 7.4 (15) | 5–6 | 7.7 (16) | 7.7 (16) | 6 | 8.2 (28) | 7.8 (26) |
| 8 | 7.9 (16) | 7.0 (15) | 7–8 | 7.4 (16) | 7.7 (16) | 8 | 8.1 (28) | 7.7 (26) |
| 12 | 6.8 (15) | 6.1 (15) | 12–13 | 6.5 (16) | 7.2 (15) | 13–15 | 5.4 (29) | 5.4 (26) |
| 15 | 5.5 (10) | 5.1 (15) | 14–15 | 5.8 (16) | 6.0 (16) | 17 | 4.6 (4) | 5.1 (7) |
| 18 | 5.3 (4) | 4.3 (13) | 19–20 | 4.7 (16) | 5.1 (14) | 20 | 3.4 (4) | 4.3 (3) |
| 22 | 4.0 (4) | 3.7 (13) | 21–22 | 4.4 (16) | 4.7 (11) | 22 | 2.8 (2) | 2.5 (2) |
| 26 | 4.5 (5)† | 4.1 (8) | 26–27 | 3.6 (13) | 5.5 (7) | | | |
| 28 | 5.4 (7)† | 3.9 (6) | 28–29 | 3.6 (11) | 5.4 (7) | | | |
| 32 | 5.4 (6) | 3.3 (6) | 33–34 | 2.9 (9) | 4.7 (7) | | | |
| 35 | 5.5 (5) | 3.1 (5) | 35–36 | 2.8 (8) | 4.3 (7) | | | |
| | | | 41–44 | 2.0 (6) | 3.1 (6) | | | |

* Numbers in parentheses give number of animals counted.
† Additional animals counted because of deaths in this group.

analyzing this factor is seen in the third group of animals of experiment 2. The infection was prevented in Rochester rats by treatment with a chemotherapeutic agent, oxophenarsine hydrochloride, Mapharsen (5 mg/kg), prior to the polonium injection and the subsequent response to polonium studied. As a preliminary check of the effectiveness of treatment, a group of animals was given Mapharsen and subsequently splenectomized. No animal developed an active bartonellosis, in contrast to 100% incidence in splenectomized animals not receiving Mapharsen. For the toxicity test 22 animals of the local colony were treated with Mapharsen and 2 days later received a dose of polonium averaging 41 $\mu$c/kg ($\pm$2.4). Their median survival time was 22 days. These data are shown in table 1, last column. The dosage in this group was slighly higher than in those not treated with Mapharsen, making the decrease in survival time difficult to interpret accurately. However, there is no evidence that prevention of the bartonellosis decreased the radiation sensitivity of the animals, and the trend is toward increasing the sensitivity of the animals to polonium. (Note that an untreated group in experiment 1 received 43.5 $\mu$c/kg [$\pm$4.1] of polonium and their median survival time was 34 days.) Thus the Rochester rat without bartonellas becomes, if anything, more like the Sprague-Dawley in sensitivity to the acute effects of polonium, and is not at all aided in coping with the radiation stress.

The Mapharsen-treated animals lost weight at about the same rate as the untreated group, but the anemia was less severe than controls, as illustrated in table 2. Bartonella bodies always appeared in the red cells of the animals not receiving Mapharsen prior to polonium exposure, while they were never definitely identified in the Mapharsen-treated group.

Hematological studies were not made on the Sprague-Dawley animals in this 2nd experiment until the 33rd day. At this time their red cell counts were 6.2 million cells/mm$^3$ (2 animals). At 35 days the average count was 5.5 (2 animals) and, at 41 to 44 days, 4.8 million cells/mm$^3$ (3 animals).

Since the Sprague-Dawley rats grow more rapidly than the Rochester-Wistar strain (they were about 85 g heavier at age of injection) and appeared to have more fat, the absolute dose of polonium to the radiosensitive tissues in these animals might have been relatively greater than expected from the nominal dosages given. To check this possibility tissue distribution studies were made on three of these animals. Comparison of the percentage of dose remaining in liver, lung, spleen, kidney, bone, muscle, lymph nodes, bone marrow, adrenal and intestine of these rats, with similar data for a large number of the local animals[3] (unpublished observations by Stannard), did not indicate differences which could account for the greater sensitivity of the Sprague-Dawley strain.

A third large experiment was conducted to differentiate, if possible, the relative roles of bartonella infection and inherent radiation sensitivity in the Sprague-Dawley strain. For this purpose, 31 Sprague-Dawley rats were infected with bartonellosis by intravenous injection of 0.1 to 0.2 ml of blood from splenectomized animals of the local colony bearing active infections. Three of these animals were splenectomized after 3 weeks and soon exhibited evidence of intravascular hemolysis and bartonella bodies in the red cells; two died as a result of infection. After approximately 6 weeks the remainder of this group and 30 uninfected animals received about 40 $\mu$c/kg (38 to 44 $\mu$c/kg

in different subgroups) of polonium chloride intravenously. The changes in red cell counts are shown in table 2 listed as experiment 3. The infected animals showed earlier anemia, episodes of gross hematuria, and bartonella bodies appeared in the red cells at about 15 days after the polonium injection. No evidence of bartonellosis appeared in the uninfected animals. The median survival time for both groups was 15.5 days; no difference in lethality was seen between infected and uninfected groups. The shapes of survival curves (not shown) were about identical also. Thus, the presence of active bartonellosis seemed to play little part in determining the acute toxicity of polonium to these animals.‡

### 2. *X-ray experiments*

The primary purpose of these experiments was to determine whether or not latent bartonellosis could be activated by X irradiation as it was by polonium injection. Thirty-two female§ rats of the local colony known to harbor the infection were exposed to 600 r as described above. Following irradiation, red blood cell counts and blood films were taken three times a week. Any animals not showing active bartonellosis during the postirradiation period were splenectomized at 40 days after irradiation. This was done to be certain they indeed carried the latent infection. Nine animals were splenectomized but not irradiated to compare the results of surgical splenectomy with radiation effects.

‡ Note also that the median survival time for Sprague-Dawley animals is again considerably shorter than for the Rochester rats in agreement with Experiments 1 and 2.

§ The choice of females for the x-ray experiment was for practical reasons. As stated earlier, no real sex differences in acute radiosensitivity have been found for the rat in this laboratory, nor would a difference in activation of bartonellosis be expected from recorded data.

The mean red cell counts in this experiment are shown in table 3. The three groups are: (a) radiated animals showing bartonella infection, (b) radiated animals never showing organisms and (c) splenectomized rats, not irradiated. The figures refer to the average of all animals in the category regardless of when the infection was manifest.

The degree of anemia occurring in the animals with active bartonellosis was greater than in those in which the infection was not activated, and its most serious manifestations could be seen to coincide with the occurrence of organisms in the blood (last column of table 3). Not shown in the mean figures, however, is the fact that the number of organisms and degree of anemia varied considerably among different animals. Three of the 32 animals died during the experiment, two had active bartonella infection at the time of death, and one did not. Further assay of mortality was not made because all remaining animals were splenectomized at 40 days as described above. Latent infection was ac-

TABLE 3.—*Effect of X irradiation on mean red cell count in Rochester rats and the appearance of bartonella organisms in the red cells.*

| Days after irradiation or splenectomy | Irradiated | | Splenectomized | Percent of radiated animals showing bartonella |
|---|---|---|---|---|
| | Bartonella positive | Bartonella negative | (Not irradiated) | |
| | Million/mm³ | Million/mm³ | Million/mm³ | Percent |
| Pre-irradiation | 8.0 | 8.2 | 7.8 | 0 |
| 3 | 7.8 | 7.4 | 6.3 | 0 |
| 5 | 6.6 | 6.7 | 3.9 | 0 |
| 7 | 5.8 | 5.9 | 3.6 | 0 |
| 10 | 5.5 | 5.3 | 4.3 | 6 |
| 12 | 4.2 | 5.5 | 4.5 | 44 |
| 14 | 3.9 | 5.3 | 4.3 | 44 |
| 17 | 4.7 | 6.2 | 4.9 | 50 |
| 19 | 5.3 | 6.2 | 4.4 | 53 |
| 21 | 5.3 | 6.1 | 4.6 | 56 |
| 24 | 5.4 | 6.2 | 4.6 | 69 |
| 26 | 5.9 | 6.6 | 4.5 | 69 |
| 28 | 5.9 | 6.5 | 4.7 | 69 |
| 31 | 6.2 | 6.7 | 5.1* | 69 |
| 33 | 6.3 | 6.8 | 5.1† | 69 |
| 38 | 6.4 | 6.9 | 4.9 | 69 |
| Number of animals | 22 | 10 | 9 | |

* 32 days.
† 34 days.

tivated by surgical splenectomy in all cases.

The nine animals which were splenectomized surgically developed anemia much more rapidly than the X-radiated animals and recovery was much delayed or incomplete. Also, bartonella bodies appeared in the blood by the 4th postoperative day in contrast to the much slower appearance after irradiation (the first radiated animal showed these on the 10th day, the last of those infected on the 25th day. The time-course after splenectomy described above agrees with findings on a larger number of splenectomized rats studied in preparation for the polonium experiments. Thus, X irradiation can activate latent bartonellosis in the rat, but not as rapidly or as regularly as surgical splenectomy. The infection is much more severe after polonium injection than after X irradiation.

## DISCUSSION

While this work was in progress Rekers[6] published evidence that X irradiation (700 to 750 r) could activate latent bartonellosis in Slonaker-Addis and Sprague-Dawley rats. The former strain carried the infection endemically, while the Sprague-Dawley were inoculated de novo when desired. Thus the X-ray data in the present experiment confirm this finding and extend it to the Wistar-derived Rochester strain. Rekers does not give detailed hematological data to allow comparison with our animals. On the other hand, he was able to obtain mortality figures after X irradiation and found a decreased mortality for this dose of X rays in the infected rats. This was true whether the animals were initially infection-free or made so by treatment with antibiotics. Qualitatively, this is parallel to our results with polonium. Recently Friedell[7] noted a relationship between bartonella infection and some radiation effects in the rat, but could find no influence on survival.

Thus all experiments available to date indicate that animals bearing bartonella infection survive the acute effects of either X irradiation or $\alpha$ particle damage as long as controls on the average, despite the more severe degrees of anemia encountered. On the other hand, it is clear that the infection may play a real part in the hematological changes following radiation.

The differences in the course of active bartonellosis produced by surgical splenectomy, polonium injection, and X irradiation require comment. In terms of severity of the anemia splenectomy produces the most drastic changes, while polonium and X irradiation produce, in the order named, less marked decreases in red cell count and slower activation of the infection. After surgical splenectomy there were recurrent periods of gross hematuria. While usually occurring at least once these recurred less frequently after polonium injection and recurrences were seldom seen after X irradiation. The peripheral blood showed evidence of active regeneration after surgical splenectomy (reticulocytosis, nucleated red cells, anisocytosis, poikilocytosis, basophilia, etc.), but this was not observed in the animals receiving polonium. There was some regeneration of red cell elements in the X irradiated animals. The explanation for the difference between surgical splenectomy and polonium-induced "splenectomy" probably lies in the relative completeness of the destruction. It is well known that polonium dis-

6. Rekers, P. E. 1951, J. Infect. Dis. **88**: 224–229.

7. Friedell, H. L. April 1952, Monthly Progress Rep., Atomic Energy Project, Western Reserve Univ., NYO-1646, 6 pp.

tributes itself rather unevenly in tissues[8] and the subsequent damage may thus be only partial. Recovery in the spleen is also irregular, with areas of degeneration in close juxtaposition to areas of repair.[9] In the case of external radiation the spleen gradually returns to normal, which might account for the more rapid recovery of the red cell count, but the delay in appearance of the organisms in peripheral blood cannot be easily explained. The destructive action of this dosage of X rays on lymphoid tissues occurs quite rapidly.

A comparison of relative dosages to the spleen from Xray and polonium in these experiments may account, in part, for the greater severity of effects with polonium. Both doses are in the range of acutely lethal doses for the whole animal, but because of the tendency of polonium to concentrate and remain in the spleen for appreciable periods, the dose to this organ is more than ten times that received in the X irradiation. Thus the total dose to the spleen when the animal receives 40 $\mu$c/kg body weight would be about 1500 rep and that received in 30 days about 6300 rep. These figures are to be compared with 600 r of 250 KV X irradiation received in the X-ray experiment. The difference in expected damage could be increased by another factor of 20 if commonly accepted figures for the relative biological effectiveness of $\alpha$ particles are considered.[10]

The experiment with Mapharsen is not completely controlled in that the Mapharsen itself may have affected the toxicity of the polonium. While further study of this point is desirable, the present conclusions are not materially affected by this possibility.

## SUMMARY

1. It has been shown that latent *Bartonella muris* infection in the rat can be activated by injecting the radioactive element, polonium$^{210}$, or by whole body exposure to 600 r of 250 KV Xrays. The anemia develops less rapidly and is not as severe as when the infection is activated by splenectomy.

2. The animals in which the infection is activated develop a more marked anemia after irradiation than uninfected animals. Despite this fact, however, the survival time after polonium was either unchanged or possibly slightly longer in the rats with active infection. This was true in the results of three types of experiment: (a) comparison of a strain of rats not carrying the disease (Sprague-Dawley) to one known to harbor the infection latently (Rochester), (b) comparison of a group of rats from the infected strain (Rochester) with a group from the same strain in which the infection was prevented by Mapharsen treatment and (c) comparison of a group from the normally uninfected strain with a group from the same strain inoculated with the disease.

3. It is concluded that the presence of an active bartonella infection in the rat does not exacerbate the lethal effects of an acute radiation dose, although the hematological response may be markedly altered.

8. Gallimore, J. C., Boyd, G. A., and Stannard, J. N. 1954, Anat. Rec. **118:** 253–274.
9. Casarett, G. W. 1952, University of Rochester Atomic Energy Project Report UR-201, pp. 167–211. (Copies may be obtained from the Reference Branch, Technical Information Service, U. S. Atomic Energy Commission, Post Office Box 62, Oak Ridge, Tennessee.)
10. N.B.S. Handbook #52, 1953. Maximum permissible amounts of radioisotopes in the human body and maximum permissible concentrations in air and water, U. S. Department of Commerce, 45 pp.

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